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CAMBRIDGE UNIVERSITY PRESS
LONDON: BENTLEY HOUSE, N.W. 1
NEW YORK: 32 EAST 57TH STREET, 22

Price 20s. net

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Iron Metabolism and Haemoglobin Formation in the Embryonated Hen Egg

3. IRON IN THE LIVER AND BLOOD OF THE HATCHING CHICK

By W. N. M. RAMSAY
Department of Biochemistry, University of Edinburgh

(*Received* 25 *October* 1951)

It is generally recognized that in many animals the liver stores an important quantity of iron at all ages. Thus, calculations based largely on figures quoted by Brückmann & Zondek (1939) and by Barcroft (1946) show that in both the adult man and the newborn lamb the liver contains roughly one-third of the non-haem iron in the body. In the course of a study which extended over both sexes of many species, Widdowson & McCance (1948) showed that at 20 weeks of age the livers of pullets contained 14·7 mg. non-haem iron/100 g. wet weight, and similar figures were obtained in pullets of 16 weeks by Chapman, Maw & Common (1950). Ramsay (1951) found that during the last few days of incubation some 200 μg. haem iron disappears from the chick embryo membranes. As there is an apparently corresponding increase in the rate of accumulation of non-haem iron in the embryo body, it seemed at first sight likely that the membrane haemoglobin was being destroyed (bile pigments also appear at this time), and that the non-haem iron liberated was being accumulated as a store. The present experiments were therefore undertaken with a view to finding out whether a significant proportion of the iron was being concentrated in the liver.

As the total amount of non-haem iron in the hatching chick (excluding the 'spare' yolk) is of the order of 350–400 μg. (Ramsay, 1951), it may be calculated that if the state of affairs is similar to that in the animals already mentioned, the liver should contain more than 100 μg. non-haem iron. McFarlane & Milne (1934) determined total iron in the liver of the chick at all stages from 9 days after the eggs were set in the incubator until 3 days after the chicks were hatched. In the hatched chick they found the liver to contain 41 μg. iron, and in the only two livers tested by the method of Hill (1931), that the non-haem iron was 52 and 66% of the total, or roughly 25 μg./liver. This figure is much less than might be expected, so it seemed important to make a re-investigation of the question. The results described in this paper establish that the analyses of McFarlane & Milne (1934) were correct and that the liver of the chick at hatching is much poorer in iron than any other liver so far studied, including that of the chicken at later stages of development.

This finding suggested the possibility that many chicks might be at least on the verge of iron deficiency, so that reliable blood-haemoglobin figures for late chick embryos and young chicks might prove interesting. As such figures did not seem to be readily available, total blood iron was determined in as many chicks as possible in the present series. It was felt that the results would be likely to afford a better estimate of haemoglobin concentration than the usual colorimetric methods, because chick blood contains nucleated erythrocytes and is often quite exceptionally lipaemic. It seemed probable that any error resulting from the presence of traces of non-haemoglobin iron in the plasma or the cells would be very much less than the pronounced errors which may be caused by turbidity in any of the simple dilution methods. The work of Rostorfer (1949), on ducks, suggests that only gasometric analyses (impracticable in this case) would give better results than total iron determinations.

EXPERIMENTAL

Newly hatched chicks from the stock of Brown Leghorn hens maintained by the Poultry Research Centre, Edinburgh, were anaesthetized with ether and bled by cardiac puncture, using an oxalated syringe and needle. The chicks were then killed by a blow on the back of the neck. The livers were removed, taking great care not to permit contamination from the 'spare' yolk, which was carefully removed from the abdomen at the commencement of the dissection. The livers were weighed and glass-homogenized (Potter & Elvehjem, 1936) with 2·5 ml. water.

The blood specimens were analysed for total Fe and the liver homogenates for total and non-haem Fe by the methods previously described (Ramsay, 1951). A volume of 0·03–0·05 ml. blood was used for each analysis, and the high catalase activity of both blood and liver made it necessary to heat all specimens to 100° before adding H_2O_2. Reduction and colour development with 2:2′-dipyridyl were as described by Ramsay (1951). In all three methods the coefficient of variation (S.D. expressed as a percentage of the mean) was of the order of 3%.

RESULTS

Blood iron. The average iron content of thirteen blood specimens was 35·5 mg./100 ml. (range 31·6–38·0, S.D. 2·0 mg./100 ml.). An isolated value of 44·5 mg./100 ml. was not included in the series. A few analyses were made on blood specimens obtained 19 days (three chicks), 22 days (two chicks) and 23 days (three chicks) after the onset of incubation, but the results all fell within the apparent normal limits for the 21st day (mean $\pm 2 \times$ S.D.). They did not suggest that any pronounced trend in haemoglobin concentration is associated with the hatching process.

Liver iron. The results of the analyses on twenty-one livers at 21 days are summarized in Table 1. The total iron averaged 45·4 μg./liver (range 32·8–85·4, S.D. 12·5 μg./liver). The percentage of non-haem iron in the total liver iron was 37–69 (mean 52 %, S.D. 8·7).

Table 1. *Iron in the liver of twenty-one newly hatched chicks*

	Liver wt. (g.)	Total iron (μg./liver)	Total iron (mg./100 g.)	Non-haem iron (μg./liver)	Non-haem iron (mg./100 g.)	Non-haem iron (% of total)
Mean	0·80	45·4	5·7	22·7	2·9	52
Range	0·56–0·94	32·8–85·4	3·8–10·5	14·4–38·4	1·7–4·7	37–69
S.D.	0·108	12·5	1·51	5·9	0·75	8·7

DISCUSSION

Blood iron. The blood-iron figures on these chicks were similar to, but generally slightly higher than, some made on adult birds from the same flock. The danger of direct comparison between birds of such very different ages is real, but it does seem likely that the chicks were not, in fact, seriously deficient in iron.

If it is assumed that the relation between total iron and haemoglobin is the same in the blood of the newly hatched chick as in that of mammals (cf. Rostorfer, 1949), it may be calculated that the observed iron concentrations correspond to haemoglobin concentrations of about 9·5–11·5 g./100 ml. Zorn & Dalton (1937) made haemoglobin determinations on similar material, but their paper unfortunately lacks the technical information which would have made a useful comparison possible. They record striking fluctuations in haemoglobin concentration during and after incubation, but do not state either the method used or the number of analyses on which their conclusions were based.

Liver iron. The total iron, at 45·4 μg./liver, agrees well with the figure of 41 μg./liver reported by McFarlane & Milne (1934). Moreover, the two isolated values which they gave for the proportion of non-haem iron in the liver fall within the limits determined in the present work. The two earlier figures were 52 and 66 %, and the mean for the present work was 52 % (S.D. 8·7 %). It is unfortunate that the present author, in a previous paper (Ramsay, 1950) expressed the view that the analyses of McFarlane & Milne (1934) were erroneously quoted. This view was based on analogy with other species, on a series of calculations in the paper of McFarlane & Milne (1934), which purported to show that during the greater part of incubation and after hatching a substantial proportion of the total iron in the body was concentrated in the liver, and on the fact that these authors failed to notice that the chick liver is actually very poor in iron. The present work emphasizes the last point, shows the analogy to be quite false, and demonstrates that the original analyses of McFarlane & Milne (1934) were correctly reported, while the calculations under discussion were erroneous.

It is interesting to note that the proportion of non-haem iron in the liver, at 52 %, is much lower than the 75 % or over which is commonly found in mammalian livers (Brückmann & Zondek, 1939; McCance & Widdowson, 1940; Scott & McCoy, 1944). It might be thought that this difference was an analytical one, as the chick livers were not washed or perfused to remove blood; they were merely dabbed with filter paper. These livers, however, are rather fatty (20–22 % ether-soluble material; Entenman, Lorenz & Chaikoff, 1940; A. K. Lough, private communication), and contain little blood. Moreover, the proportion of non-haem iron was not less than usual in livers which contained much haem iron (and might therefore have been contaminated with larger volumes of blood). The difference is therefore probably a real one, and would be expected from the knowledge that at this stage the chick liver is not carrying a large store of non-haem iron.

Other chicks analysed at the same time gave figures for the quantity and distribution of iron similar to those reported in the previous paper (Ramsay, 1951). Calculations based on the figures in the present work and on values taken from the graphs given by Ramsay (1951) show that the non-haem iron in the liver amounts to no more than 2·0–2·5 % of the total iron in the body or 6–8 % of the non-haem iron. Recalculation from the analyses of McFarlane & Milne (1934) shows their figures to

be in good agreement with the present ones in this respect also, except that they did not make determinations of non-haem iron in the whole chick. This state of affairs may be in part a reflexion of the small size of the liver, which at hatching amounts to only 2–3 % of the body weight, while according to Hamilton, Boyd & Mossman (1945) the human liver at birth may be 5 % of the body weight. The difference in proportion of functional tissue weight may be very much greater than these figures suggest because, as has already been noted, fully one-fifth of the wet weight of the chick liver consists of ether-soluble material. This particular problem in comparative biochemistry would seem to be an ideal one for attack by the method advocated by Vendrely & Vendrely (1949) and Davidson & Leslie (1950), in which deoxyribonucleic acid determinations are made and the number of cells calculated from the results with the aid of certain fundamental assumptions.

The question arises whether iron normally stored in the liver is displaced in the hatching chick to other sites, or whether a very large proportion of the non-haem iron in the body is to be regarded as belonging to the 'parenchymatous iron' fraction (Hahn & Whipple, 1936) which is not believed to be readily available for haemoglobin formation. Neither the kidney analyses of McFarlane & Milne (1934) nor a few spleen analyses made in this laboratory suggest that these organs usurp the function of the liver in respect to iron storage. The idea is tempting that the organism does not develop such functions until the 'spare' yolk is exhausted. This does not happen until about a week after the chick is hatched.

The problem remains of the fate of the iron which may be liberated by haemoglobin catabolism towards the end of incubation. Whatever may be the solution, it is certain that iron liberated in this way is not 'stored' in the sense in which that word is usually used in relation to iron metabolism.

SUMMARY

1. Total blood iron in thirteen newly hatched chicks was 35·5 (S.D. 2·0) mg./100 ml.

2. The livers of twenty-one newly hatched chicks contained only 45·4 (S.D. 12·5) μg. total iron and 22·7 (S.D. 5·9) μg. non-haem iron.

3. It is concluded that the liver of the newly hatched chick contains no appreciable store of iron.

The author is indebted to the Poultry Research Centre for the supply of eggs, to the Agricultural Research Council for a grant from which the expenses of the work were defrayed, and to Mr G. R. Wilson for technical assistance.

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Studies in Rhodopsin

4. PREPARATION OF RHODOPSIN

By F. D. COLLINS, R. M. LOVE and R. A. MORTON
Department of Biochemistry, The University of Liverpool

(*Received* 18 *August* 1951)

The history of visual pigments has been reviewed on numerous occasions—see Collins & Morton (1950) and Wald (1951). In recent years the methods used in preparing solutions of rhodopsin have involved at least partial separation of rod outer segments. If retinas are shaken vigorously in saline or other suitable suspending medium the rod outer segments become detached. Various methods have been used for separating these 'rods' from the remainder of the retinal suspension. Lythgoe (1937) poured the suspension through fine wire gauze and showed that the rods, pigment granules and a few isolated cells passed through, but that the main mass of nuclear and fibrous material was held back. Krause & Sidwell (1938) centrifuged the retinal suspension and found that the rods formed a layer on top of the other retinal fragments which could then be scraped off. Saito (1938) used 40–45 g. sucrose/100 ml. water as the suspending medium and found that on centrifuging, the rods remained in suspension. The rods could be thrown down when diluted with sufficient saline. The method has been used by Collins & Morton (1950), by Bliss (1950) and by Wald (1951). In addition, Wald (1949) has often simply scraped the rods off the retina with a fine spatula or forceps.

The purity of solutions of rhodopsin prepared from rods isolated by any one of the methods described above varies greatly. With frog retinas it is easier to obtain pure solutions than with cattle retinas. It was the purpose of the present work to investigate more fully the reasons for these differences and to develop improved techniques applicable to cattle retinas which can be obtained from abattoirs in large numbers. Comparatively large amounts of cattle rod outer segments would greatly facilitate future work.

EXPERIMENTAL

Solutions

Potash alum $K_2SO_4.Al_2(SO_4)_3.24H_2O$, 4% (w/v) in water.

Digitonin solution. 1 or 2% (w/v) in water; prepared by heating the mixture of digitonin and water to boiling until solution occurs and then quickly cooling.

Buffer solution, pH 9·3. $Na_2B_4O_7.10H_2O$, 19 g./l.

Formaldehyde (neutral). A 40% (w/v) solution of formaldehyde was shaken with $MgCO_3$, allowed to stand overnight and filtered.

Animals

Eyes were obtained from freshly killed cattle and transported from the local abattoir to the laboratory in closed tins. The dissection, carried out in red light, has been described by Collins & Morton (1950).

The frogs used were *Rana esculenta*.

Absorption measurements

All measurements were made with a Beckman photoelectric spectrophotometer. A 1 cm. cell was used. The compensating cell contained a solution of digitonin and buffer equivalent in strength to that used for the rhodopsin solutions. The cells were filled in the dark room (using red light), placed in the cell holder and covered with a black cloth. They were then carried to the Beckman and placed in position in the light-tight cell space. Although rhodopsin solutions are sensitive to light no noticeable photodecomposition took place during measurements.

Criteria of purity

The spectroscopic purity of rhodopsin solutions has been assessed as follows. The ratio of the extinction at wavelength λ to that at $\lambda_{max.}$ (about 500 mμ.) has been designated P_λ. It is found that in the presence of absorbing impurities P_λ is higher than that for pure rhodopsin. Hence as the degree of purity increases P_λ will tend to a constant low value. Usually P_λ has been measured at 400 mμ., a minimum in the rhodopsin curve (cf. Collins & Morton, 1950). The lowest value so far obtained for P_{400} is about 0·25. As rhodopsin solutions have an absorption maximum near 275 mμ. due to the presence of protein (tyrosine and tryptophan) and also some nucleotide absorption near 260 mμ. (Collins, Love & Morton, 1952) it has been found useful to record P_{275} in some cases.

RESULTS

Preliminary investigations

Separation of rods by scraping. Wald (1949) separated rods by carefully scraping the surface of the retina with a fine spatula. This method was investigated.

Cattle retinas were spread out flat on a piece of filter paper with the rods uppermost. The surface was then gently stroked with a coarse hair brush, a spatula having been found useless, and the brush shaken at frequent intervals in saline solution. The saline suspension, when examined microscopically, was seen to contain large fragments of tissue, rods and blood cells. In another experiment a fine camel-hair brush was used and microscopical examination revealed a suspension of rods and blood cells only. In both

cases a sugar separation (see next section) was subsequently carried out, the rods hardened in alum and finally extracted with aqueous digitonin solution. The solutions yielded values of P_{400} of 0·53 and 0·68 respectively.

These results and the extra time required made this method unsuitable for cattle retinas.

Crude rod suspensions. These can be obtained by shaking a number of retinas in saline or sucrose solutions and pouring through a fine wire gauze (60-mesh). The resulting suspension contains most of the rods but is heavily contaminated with other tissue fragments. Lythgoe (1937) pointed out that frog retinas do not fragment during shaking, apart from releasing the rod outer segments, but cattle retinas as a whole appear to break up into small pieces. This we confirmed. Frog and cattle retinas were shaken vigorously in saline. The suspension of frog 'rods' was almost uncontaminated by other tissue fragments, but the suspension of cattle rods contained many blood cells and a high proportion of unwanted tissue and melanin pigment granules. The frog rod outer segments were much the larger.

Separation of rods by Saito's method

Saito's method, as modified by Collins & Morton (1950), was used at first. However, the purity of cattle rhodopsin solutions obtained by this method was very variable and the need for improvement soon became apparent.

The original method was as follows. The cattle retinas, dissected in red light, were shaken vigorously in 1·32 M-sucrose for 30 sec. using 15 ml. sucrose solution per twenty-four retinas, and poured through the fine wire gauze. The resulting suspension was centrifuged at 1600 *g* for 15 min. At the end of that time it could be seen that the fibrous part of the retina, blood cells and melanin were thrown to the bottom of the tube, while the rods remained suspended in the supernatant. This was decanted, diluted with 2–3 vol. of 0·9% (w/v) saline and centrifuged until clear. The brilliant scarlet precipitate was hardened in 4% (w/v) alum solution for 1 hr. The rods were then centrifuged down and washed once with saline, and finally extracted for 1 hr. with 1 ml. of 1% (w/v) digitonin solution and centrifuged. The clear supernatant was mixed with 1 ml. of buffer solution (pH 9·3), recentrifuged, and the absorption spectrum measured.

The following variables were investigated:

(i) The effect of pH on the digitonin extraction. The least amount of contaminating impurities were extracted at an acid pH, but a fine precipitate tended to form which could not be centrifuged down. Alkaline extracts were very impure. Hence a neutral extractant was employed.

(ii) The concentration of sugar seemed unimportant between about 1·0 and 1·6 M, but below 0·88 M the rods sank to the bottom and formed a layer on the debris, while above about 1·6 M the whole brei floated.

(iii) Re-extraction of the retinal debris with 1·32 M-sucrose yielded a less pure fraction. More rods were obtained, but with four successive 'sugar extractions' the purity deteriorated thus: $P_{400}=0·446$, 0·53, 0·733 and 0·858.

(iv) The duration of the alum treatment is apparently unimportant provided it exceeds 30 min. If the rods are left for periods greater than 24 hr. at room temperature there is a slow loss of rhodopsin.

(v) The extraction by means of digitonin solution is nearly complete in 1 hr., but thereafter increasing amounts of impurities go into solution.

Attempts to remove impurities from rods. Rods separated by Saito's method were extracted with an alkaline buffer (pH 9·3) followed by an acid one (pH 4·1). The rods were then hardened in alum and extracted as usual; $P_{400}=0·477$. In other experiments the rods were extracted with 0·1% Dispersol A (an Imperial Chemical Industries Ltd. detergent); untreated rods gave a value of $P_{400}=0·783$, whilst after treatment P_{400} became 0·565. Further experiments showed that only very heavily contaminated rod preparations were improved.

The following organic solvents were tried: phenol, acetone, amyl alcohol, pyridine, *cyclo*hexane, ethyl acetate and ethyl butyrate. All except the last bleached the rhodopsin immediately—ethyl butyrate did so in about 24 hr.

It was noticed that rods spun down after having been separated were contaminated with many black particles. Attempts were made to separate these small particles using a fine spatula. A typical experiment was as follows: the rods were divided into two portions: (1) was used as a control and (2) was separated into (2*a*) rods relatively free from black particles and (2*b*) rods contaminated with black particles. The values of P_{400} were respectively 0·282, 0·312 and 0·362. In another experiment the 'clean' rods gave $P_{400}=0·30$ and contaminated rods gave $P_{400}=0·37$. The conclusion to be drawn from this experiment was that even the 'cleaned' rods gave less pure solutions than those obtained from untreated rods.

Treatment with formaldehyde. Although ordinary formaldehyde solutions (40%, w/v) at once destroyed rhodopsin it was found that a neutralized solution (i.e. left to stand with magnesium carbonate overnight) did not. It was found that solutions of rhodopsin prepared from rods treated with formaldehyde had a value of P_{275} lower than had been obtained previously and that 10 min. in formol was the optimum time of treatment (Tables 1 and 2). Good results were obtained by combining formol treatment with differential centrifugation (see below) in winter and spring, but the results in summer were unsatisfactory (Table 2). The purest cattle rhodopsin solution obtained by this method had $P_{400}=0·236$, $P_{275}=2·05$ at pH 9·3 (Fig. 2). Later experiments have shown that it is better to treat with alum before the formol treatment.

Attempts to remove impurities from rhodopsin solutions. Various attempts were made to precipitate the rhodopsin from its digitonin solution in the hope that impurities would be left in solution. Acetone was tried first; this solvent destroys rhodopsin at room temperature but not at 0°. However, it was found that all the protein present was precipitated and that no purification resulted. Addition of acetone in steps might be more successful if combined with facilities for low temperature centrifugation.

Sodium sulphate and magnesium sulphate and sodium chloride were next tried, but did not result in any improvement when the precipitated rhodopsin was redissolved. Ammonium sulphate was more successful.

Table 1. *Effect of duration of treatment of rods with formaldehyde on purity of rhodopsin*

(Cattle rod outer segments were treated with neutralized formaldehyde solution for various periods and then treated with alum and extracted with digitonin solution as usual. The data refers to the absorption spectra of the resulting solutions.)

Time in formol (min.)	P_{400}	P_{275}	$E_{500m\mu}$
	First experiment		
0	0·322	3·3	0·251
10	0·318	2·45	0·277
20	0·322	2·55	0·192
40	0·350	2·88	0·148
17 hr.	About half of the rhodopsin destroyed		
	Second experiment		
0	0·330	3·64	—
5	0·295	2·45	—
10	0·295	2·11	—
15	0·300	2·18	—

original solution. The second fraction contained a little rhodopsin of low optical purity. Fractions 3 and 4 contained no rhodopsin, but had maxima near 270 mμ.

These results showed that rhodopsin solutions contained irrelevant material absorbing at 275 mμ. and that some of this impurity could be removed by fractional precipitation with ammonium sulphate. Better results were obtained later by another method.

Modifications of Saito's method

Differential centrifugation. Rods from twenty-four ox eyes were prepared by Saito's method. The sugar suspension was diluted with 3 vol. of saline and centrifuged for 2 min. only at 1200 ***g***. The precipitate was treated with alum and the supernatant cleared by centrifuging for 15 min. at 1800 ***g***, and this second precipitate was also treated with alum. When the precipitates were extracted, the values of P_{400} were 0·28 and 0·38 respectively. The value of P_{275} for the first solution was 2·29.

It was found that for this method to be effective the eyes had to be dissected and extracted very soon after death. Another disadvantage was the low yield—less than 20 % of the total rhodopsin/retina.

Table 2. *The effects of treating rod outer segments with formaldehyde solution on purity and yield of rhodopsin*

(Cattle rod outer segments were treated with neutralized formaldehyde solution for 10 min., then with alum for 1 hr. and extracted as usual with digitonin solution. The data refer to the absorption spectra of the resulting solutions.)

Solutions prepared in the winter

	Formol treatment			No formol treatment		
	P_{400}	Yield*	P_{275}	P_{400}	Yield*	P_{275}
Mean	0·303	0·056	2·80	0·293	0·066	3·84
Standard error	0·0089	0·0075	0·15	0·0096	0·0064	0·33
No. of samples	19	18	19	13	12	14
Range	0·236–0·39	0·02–0·14	2·05–4·9	0·217–0·34	0·03–0·10	2·8–7·2

Solutions prepared in the summer

	Formol treatment		No formol treatment	
	P_{400}	P_{275}	P_{400}	P_{275}
Mean	0·515	6·9	0·324	4·1
Standard error	0·0605	0·87	0·017	0·29
No. of samples	11	10	11	9
Range	0·26–0·93	2·9–11·3	0·27–0·44	2·95–5·9

* Yield $= \dfrac{\text{(Extinction at 500 m}\mu\text{. due to rhodopsin)} \times \text{(vol. of soln.)}}{\text{(No. of retinas)}}$.

Rods from fifty cattle eyes were extracted three times with 1 ml. of 0·5 % digitonin solution and the combined extracts were mixed with an equal volume of buffer, pH 4·5. The resulting solution had $P_{400} = 0{\cdot}575$, $P_{275} = 7{\cdot}7$. On adding increasing amounts of solid ammonium sulphate four fractions were obtained. The first contained most of the rhodopsin, P_{275} and P_{400} both being lower than in the

The sugar gradient method. The combination of differential centrifugation and extreme freshness of the eyes in the Saito method produced many solutions of cattle rhodopsin purer than had ever been obtained before, but in view of the low yield and the difficulty experienced in obtaining sufficiently fresh eyes a new modification was developed and is described below.

Retinas are shaken vigorously with about half their volume of 0·9% (w/v) saline in order to detach the rod outer segments. The mixture is stirred into a piece of 60-mesh brass gauze shaped in the form of a hollow until all the liquid has passed through, whereupon the retinal debris is transferred to a glass tube and shaken again with saline. This treatment is done four times, after which the retinal fibrous mass becomes considerably reduced in bulk and is usually colourless. The resulting suspension contains tissue fragments, blood, melanin and rods. It is transferred to a centrifuge tube, which should not be more than three-fifths filled by it. Saturated sucrose solution—greater than 2·0M—is now carefully poured down the side of the tube so as to form a lower layer of about half the volume of the saline

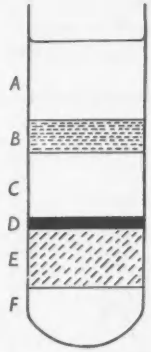

Fig. 1. This diagram shows the disposition of the various tissue fragments after being centrifuged in sucrose solution in which a density gradient had been established as described in the text (p. 295). *A*, pale opalescent solution containing some protein; *B*, dense, deep scarlet, layer of rod outer segments; *C*, faintly pink opalescent layer containing a few rod outer segments and some small tissue fragments; *D*, dense, dark red, layer containing mostly red blood cells; *E*, densely packed layer, containing the greater bulk of the disintegrated retina as well as melanin granules; *F*, colourless, almost saturated, sucrose.

suspension. The next part of the process requires practice. A flat-ended glass rod is used, and with it the saline-sugar interface is stirred. The object is to produce a gradient of concentrations of sucrose down the tube, ranging from zero, i.e. saline only, at the top, through gradually increasing concentrations, to saturated sucrose at the bottom. The tube is then centrifuged at 1800 *g* for 15–20 min. On removing the tube from the centrifuge, all the components of the suspension are found to have accumulated in their own pycnotic level.

Fig. 1 shows a diagram of an actual separation. The various zones are sucked off using a tube drawn out at the end and turned up so as to resemble a crochet hook. The rods are diluted with 2–3 vol. of saline and centrifuged down.

It was found that sucrose solutions in a series of strengths from 0·76 to 1·17M at 0·06 intervals could, by careful pipetting, be placed one on top of another in a centrifuge tube without mixing appreciably. The strongest ones were, of course, on the bottom, and the boundaries between the solutions could be clearly seen. Their positions were then marked. The crude saline suspension of rods was pipetted on top of them all and the whole centrifuged. It was found that cattle rods are isopycnotic with 0·88 (± 0·15)M-sucrose, while the blood cells, etc., come to rest in sucrose of greater than 1·0M. This being so, it was hoped that, by making a series with a deep 0·94M layer, the rods could be separated a long way from the blood cells. The idea was eventually abandoned, however, since once the technique had been learned the stirring method was far less tedious. The rods from frogs appear to be denser than cattle rods.

Table 3. *Variations in the purity and yield of rhodopsin solutions from rod outer segments prepared by the sugar gradient method*

(Cattle rod outer segments were separated by flotation in sucrose solution with a density gradient. The isolated rods were treated with alum and extracted with digitonin solution. The data refer to the absorption spectra of the resulting solution.)

	P_{400}	P_{275}	Yield*
Mean	0·293	3·83	0·212
Standard error	0·0061	0·13	0·038
No. of samples	16	14	11
Range	0·26–0·34	3·1–4·5	0·05–0·49

* Yield $= \frac{(\text{Extinction at 500 m}\mu\text{. due to rhodopsin}) \times (\text{vol. of soln.})}{(\text{No. of retinas})}$.

If the rod preparation was not very pure, then a repetition of the gradient process effected an improvement, but further treatments only worsened the preparation, due, no doubt, to excessive handling of the rods. In any second purification, the top layer was usually water-clear, but a little retinal tissue could be seen under the rod 'band'.

Table 3 shows that the yield of rhodopsin per retina is greatly improved and that the values of P_{400} indicate fairly good purity.

For these procedures it was unnecessary to use extremely fresh eyes. On one occasion 130 eyes were treated in one day and, although the time taken for dissection and treatment was considerable, the value of P_{400} for an extract of some of the resulting rods was 0·30.

Microscopic examination of rod suspensions in the course of various treatments

No staining or fixing was used.

(*a*) Sugar separation. (i) Crude rod suspension. Rods were mostly intact with many red blood cells and other, not easily distinguished, tissue fragments present. (ii) After a sugar separation. Some rods were deformed, but the majority were unchanged. Very few other tissue fragments were present. (iii) After another sugar separation. Very few intact rods remained.

(*b*) Effect of time on a rod suspension in 0·9% (w/v) NaCl. (i) Fresh—rods remained intact. (ii) 0·5–1 hr.—some changes were noticed. (iii) 1–2 hr.—many deformed and broken rods were seen. (iv) 3 or more hr.—very few intact rods remained.

(*c*) Effect of various reagents on rod suspensions. (i) Formaldehyde (40% neutralized)—the rods were seen to curl up forming almost complete circles and sedimented readily. (ii) Digitonin solutions—the rods were broken up completely. (iii) Alum solutions (4%, w/v)—the rods were broken up completely and sedimented quickly. (iv) A solution containing 2% formaldehyde and 0·9% KCl—the same changes occurred as described under (*b*) but a little more slowly.

DISCUSSION AND CONCLUSIONS

Separation of rod outer segments

The separation of rod outer segments from the other tissue fragments present in the initial crude suspension depends on two factors, the size and the density of the rods. The rods are fairly large (about $3 \times 30\ \mu$.) and sediment rapidly in water even if the centrifugal force is as low as $600\ g$. In this respect they are comparable to red blood cells and nuclei. However, the rods differ from blood cells and nuclei in one important property, they are less dense and will float in 0·88 M-sucrose (sp.gr. = 1·114).

If these were all the facts then the methods described would separate rods from all tissue fragments except (*a*) particles isopycnotic with rods, and (*b*) particles so small that, at the centrifugal forces used (about $1800\ g$), they are removed too slowly from the rod zone. A second separation should enable the majority of small particles not isopycnotic with the rods to be eliminated. This presupposes that rod outer segments are stable. However, microscopic examination reveals quite clearly that they are not and that they fragment. This explains why a repetition of a sugar separation does not result in any great improvement. As the rods break up greater centrifugal forces would become necessary to effect a separation. This was not considered practical, and the writers prefer one sugar separation performed as quickly and as efficiently as possible. Subsequent treatments with alum and neutralized formaldehyde yield solutions which are very pure.

A recent study, using the electron microscope, of rod outer segments by Sjöstrand (1949) indicates that rods are composed of several thousand disks each about 3 mμ. thick. He records the fact that rods when broken up yield fragments which may contain a few disks or many hundreds. This is in good agreement with our own observations and reveals still more clearly the difficulties encountered in attempting to separate rod outer segments.

The effect of this fragmentation of rods will be manifested in two ways. The first will be the increased difficulty of effecting an efficient separation from other tissue fragments. The second and perhaps more serious will be the presence of submicroscopic particles (fragments containing only a few 'disks') which will not be centrifuged out after the digitonin extraction. This will make the final rhodopsin solution turbid.

Characteristics of pure (?) rhodopsin

In the absence of any precise chemical criterion of purity spectroscopic ones are used instead. A value of P_{400} below 0·24 to 0·26 has not yet been obtained (cf. Wald, 1951). Similarly, a value of P_{375} of less than 2·05 has not been obtained. The curve shown in Fig. 2 represents such an absorption curve and it is worth while to examine it in detail.

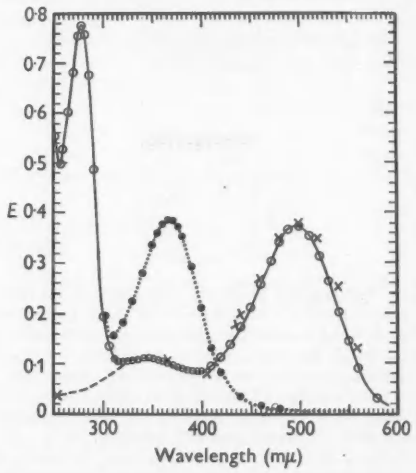

Fig. 2. –○—○–, absorption spectrum of a very pure solution of cattle rhodopsin, pH 9·2; × × ×, relative photochemical efficiencies. $[(\epsilon_\lambda \times \gamma_\lambda)/\epsilon_{500\ m\mu} \times \gamma_{500\ m\mu}$ multiplied by a factor so that the two curves correspond at 500 mμ.; after Schneider *et al.* (1939)]. . . .●. . . .●. . . , absorption spectrum of bleached solution (cf. Collins & Morton, 1950); – – – –, extrapolation indicating probable absorption of the rhodopsin chromophore below 320 mμ.

In Fig. 2 it is shown that a very close correspondence exists between the rhodopsin absorption curve, and the relative photochemical efficiency curve of Schneider, Goodeve & Lythgoe (1939). The last curve predicts a small peak near to 350 mμ. which is in fact found. It also predicts that at 254 mμ. the absorption of the rhodopsin chromophore should be only about 6% of that at 500 mμ. A plausible extrapolation for the absorption due to rhodopsin chromophore is shown in Fig. 2 by means of dashes. The difference between this and that actually found

will be due to the protein containing tyrosine and tryptophan and can be calculated as follows. If E_{280} represents the total extinction at 280 mμ. after subtracting the absorption due to the rhodopsin chromophore, and E_{290} is the corresponding value at 290 mμ. then

$$E_{280} = x + y,$$
$$E_{290} = 0{\cdot}448x + 0{\cdot}710y.$$

Here x = extinction at 280 mμ. due to tyrosine and y = extinction at 290 mμ. due to tryptophan, both at pH 9·2. (Collins & Morton, 1950, give similar equations; the difference is due to the fact that a purer sample of tryptophan was obtained for the present work.)

animal proteins is of the same order; casein (6·6 and 1·2), cattle fibrin (6·5 and 3·0), haemoglobin (3·2 and 1·3) serum albumin (4·8 and 0·5) and serum globulin (6·7 and 2·3).

It should be pointed out that the conclusion that the only absorbing materials present are the rhodopsin chromophore, tyrosine and tryptophan does not exclude small amounts of phenylalanine, nucleotides or other unidentified materials absorbing in the region 250–300 mμ.

It is not yet possible to say if the rhodopsin solutions now obtainable are 'pure', but they are in very close agreement with the results obtained by Wald and his co-workers (Wald, 1951). It seems likely that any contaminants present show no

Table 4. *Analysis of the ultraviolet absorption of a rhodopsin solution*

(Solution in 1% (w/v) aqueous digitonin and borate buffer, pH 9·2.)

λ (mμ.)	(1)	(2)	(3)	(4)	(5)	(6)	(7)
300	0·199	0·076	0·123	0·085	0·052	0·137	−0·014
290	0·491	0·058	0·433*	0·127	0·305	0·432	+0·001
285	0·681	0·053	0·628	0·214	0·370	0·584	+0·044
280	0·764	0·051	0·713*	0·283	0·430	0·713	0·000
275	0·753	0·047	0·706	0·297	0·415	0·712	−0·006
270	0·681	0·044	0·637	0·252	0·398	0·650	−0·013
265	0·600	0·042	0·558	0·213	0·344	0·557	+0·001
260	0·529	0·041	0·488	0·188	0·282	0·470	+0·018
255	0·498	0·040	0·458	0·235	0·216	0·451	+0·007
250	0·552	0·040	0·512	0·382	0·164	0·546	−0·034

Extinction due to: (1) Actual rhodopsin solution.
(2) Rhodopsin chromophore.

Extrapolation making the assumptions mentioned in text (p. 297).

(3) Difference between (1) and (2).
(4) Calculated absorption due to tyrosine.
(5) Calculated absorption due to tryptophan.
(6) Sum of (4) and (5).
(7) Difference between (3) and (6).

* (4) and (5) were calculated from these figures using the equations given in the text (p. 297).

In Table 4 is set out the results of an analysis of the curve shown in Fig. 2. The absorption due to tyrosine and tryptophan at 280 mμ. has been calculated and, from the curves of pure tyrosine and tryoptophan, the absorption due to these substances has been calculated at other wavelengths. When this absorption, calculated to be due to tyrosine and tryptophan, is subtracted from the protein curve (= rhodopsin less absorption due to the rhodopsin chromophore) the differences are small and are both positive and negative. It may be concluded that, within these limits, the absorption of rhodopsin can be accounted for in terms of the rhodopsin chromophore, tyrosine and tryptophan. If one assumes the data given by Collins & Morton (1950) for the $E^{1\%}_{1\,\mathrm{cm.}}$ of rhodopsin (= 6·6 at 500 mμ.), tyrosine (= 78 at 280 mμ. at pH 9·2) and tryptophan (= 262 at 280 mμ. at pH 9·2) the approximate amounts of tyrosine and tryptophan as a percentage of the dry weight of rhodopsin are 6 and 3 respectively. The percentage of tyrosine and tryptophan in other absorption in the region 250–700 mμ. and that the solutions are sufficiently pure for chemical analyses to be meaningful.

SUMMARY

1. Various methods for the separation of rod outer segments from cattle retinas have been investigated, attention being paid to the optical purity of the rhodopsin solutions obtained by extraction of the rod outer segments.

2. A new method has been developed depending on the fact that cattle rods have the same density as 0·88 M-sucrose. The rods are separated by centrifuging them in a sucrose solution in which a density gradient has been established.

3. Various treatments of the isolated rods have been tried in order to eliminate impurities. The best results have been obtained by treating with 4% (w/v) alum solution for 1 hr. followed by 40% (w/v) neutralized formaldehyde solution for 10 min.

4. The effects of these various treatments on the appearance of the rods has been followed microscopically.

5. Very pure cattle rhodopsin solutions have been obtained showing maxima at 498, 345 and 275 mμ. The last band can be accounted for by the presence of tyrosine and tryptophan only. Making some plausible assumptions, rhodopsin appears to contain 6% tyrosine and 3% tryptophan on the dry weight of the protein.

We would like to thank the Medical Research Council for grants. One of us (F.D.C) participated in this work as a holder of an Imperial Chemical Industries Research Fellowship.

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Estimation of Protamine and Insulin in Protamine Zinc Insulin

By F. A. ROBINSON and KITTY L. A. FEHR
Research Division, Allen and Hanburys Ltd., Ware, Hertfordshire

(*Received* 5 *September* 1951)

In a recent publication, Franklin & Quastel (1950) reported that mixtures of proteins could be separated into their individual components by paper chromatography using solutions of sucrose or sodium potassium tartrate for development. The difficulty of locating the position of the protein zones was overcome by adding haemin to the protein mixture and then streaking the paper with a solution of benzidine and hydrogen peroxide. Jones & Michael (1950) separated proteins on columns of cellulose by development with buffer solutions containing ammonium sulphate and located the position of the individual components by treatment with suitable dyestuffs which stained the zones occupied by the proteins, but did not give coloured spots with amino-acids or peptides. More recently, Papastamatis & Wilkinson (1951) used bromothymol blue and tetrabromophenolphthalein ethyl ester to indicate the position occupied by proteins in paper chromatograms. The publication of this last paper prompts us to report our own work on the chromatographic separation and estimation of protamine and insulin.

The initial experiments on the separation of insulin and protamine by paper chromatography were carried out with strips of Whatman no. 1 filter paper 2 cm. wide, using 10 μl. spots of a solution containing 1% protamine sulphate and 1% insulin (w/v). The strips were developed in a downwards direction in the usual way according to the method of Consden, Gordon & Martin (1944) with the phases of a mixture (by vol.) of *n*-butanol (40%), glacial acetic acid (10%) and water (50%), a mixture generally used in the paper chromatography of amino-acids. Solway purple, as recommended by Jones & Michael, was used for indicating the position of the protein bands. Two bands were formed, one due to protamine at the point of application and the other due to insulin, some 6 cm. below the point of application; the solvent front travelled about 25 cm. The separation was not entirely complete, however, and a number of other solvent mixtures were tried. Better results were eventually obtained with the upper phase obtained by equilibrating a mixture of *n*-butanol and glacial acetic acid (3:1 by vol.) with an equal volume of water. This resulted in the formation of two well-defined bands with R_F values of 0 and 0·43 respectively, and this mixture was used in all subsequent work.

The estimation of substances separated by paper chromatography often presents considerable difficulty, although fairly satisfactory methods of estimating individual amino-acids on paper chromatograms have recently been described. It occurred to us that a protein might be estimated with a fair degree of accuracy by the method of retention analysis described by Wieland & Fischer (1948). These workers showed that if a spot of an amino-acid solution was placed near the edge of a

sheet of filter paper and the paper then dipped into a solution of a cupric salt, the latter was drawn up by the paper and cupric ions became uniformly distributed throughout the paper except in the area immediately above the amino-acid spot. In this region no copper could be detected on subsequently spraying with a suitable indicator, because the amino-acid retained the copper ions in the form of a complex, thus preventing them from passing further up the paper. The area of the white copper-free wedge formed above the spot was proportional to the amount of amino-acid present. It was thought possible that the same principle could be applied to the estimation of a protein using instead of a copper salt a solution of a dyestuff with an affinity for the particular protein concerned.

To test this hypothesis spots of different sizes of solutions of insulin, protamine sulphate, blood albumin and edestin were applied to rectangular sheets of filter paper, and the filter papers dried and dipped into solutions of Solway purple or erythrosine. The dyes were allowed to rise about 5 cm. above the protein spots. In every instance a wedge-shaped area above the protein spot remained white and the areas of these wedges were approximately proportional to the amount of protein present.

Attempts were then made to apply retention analysis to mixtures of protamine sulphate and insulin after separation by paper chromatography. A spot of the solution to be tested was applied at one corner of a rectangular sheet of filter paper. The paper was developed along the longer axis with the aqueous butanol-acetic acid mixture and dried. It was then cut in such a way as to separate the protamine which remained at the point of application from the insulin spot which had moved about 10 cm. away from it. Both halves of the paper were then dipped in a dye solution until the dye had risen about 5 cm. above the protein spot. Estimates of the amount of protamine calculated from the area of the unstained wedge above the protamine spot were in fair agreement with the amount of protamine known to be present, but it was impossible to estimate the amount of insulin in a similar manner because the insulin spot, in contrast to the protamine spot, was very diffuse, marked 'tailing' generally having occurred. All attempts to sharpen the insulin zone so as to make it amenable to retention analysis have been unsuccessful.

At this stage we learnt that Dr W. Dickinson had used bromocresol green for indicating the position of different protein bands in paper chromatograms of crude insulin solutions and had succeeded in eluting the insulin bands from such paper chromatograms by means of a borate buffer solution of pH 9·2; the colour of the eluate was proportional to the amount of insulin present. We therefore investigated this method and found that we obtained satisfactory results for the estimation of insulin after chromatographic separation from protamine. A number of other dyes were examined, but none gave better results than bromocresol green.

EXPERIMENTAL

Separation of insulin from protamine by chromatography

In preliminary experiments 10 μl. drops of a 1% (w/v) protein solution, as well as a mixture of protamine sulphate and insulin solutions, were placed on Whatman no. 1 filter-paper strips (2 cm. broad) and developed with a large number of solvent mixtures. Each of the protein solutions when examined separately gave single spots. The protamine sulphate was derived from salmon milt and the insulin from ox pancreas.

The mixture found to be most satisfactory for the separation of protamine from insulin was the upper phase obtained by equilibrating *n*-butanol, glacial acetic acid and water (3:1:4 by vol.). This solvent mixture gave a clean separation of protamine and insulin with R_F values of 0·00 and 0·43–0·45 respectively.

Retention analysis of proteins

Preliminary experiments were carried out with solutions of insulin, protamine sulphate, blood albumin and edestin, varying the concentrations and volumes of the solutions used. A drop of each solution was placed 2 cm. from the lower edge of a rectangular piece of Whatman no. 1 filter paper, which was suspended vertically in a covered beaker so that the lower edge of the paper dipped about 1 cm. into the dye solution. The dye was allowed to rise about 5 cm. above the protein spots, and the paper was then dried. The white portion above the spot was traced on to graph paper and its area measured.

Using a 0·1% (w/v) solution of erythrosine, the results recorded in Table 1 were obtained. Fig. 1 illustrates the

Table 1. *Effect of concentration and drop size on the retention analysis of protein solutions with erythrosine*

(For experimental procedure see text.)

Protein	Concentration of solution (%, w/v)	Volume of drop (μl.)	Retention area (sq.mm.)	Relative retention areas
Insulin	1	5	61	1·0
	1	10	102	1·7
	1	20	202	3·3
Protamine sulphate	1	5	99	1·0
	1	10	198	2·0
	1	20	356	3·6
Protamine sulphate	0·2	5	81	1·0
	0·4	5	180	2·2
	0·6	5	285	3·5
	0·8	5	365	4·5
Blood albumin	0·125	10	55	1·0
	0·25	10	114	2·1
	0·5	10	167	3·0
Edestin	1	10	18	1·0
	2	10	38	2·1

appearance after retention analysis of filter papers to which 5 μl. spots of 0·2, 0·4, 0·6 and 0·8% (w/v) protamine sulphate solutions had been applied.

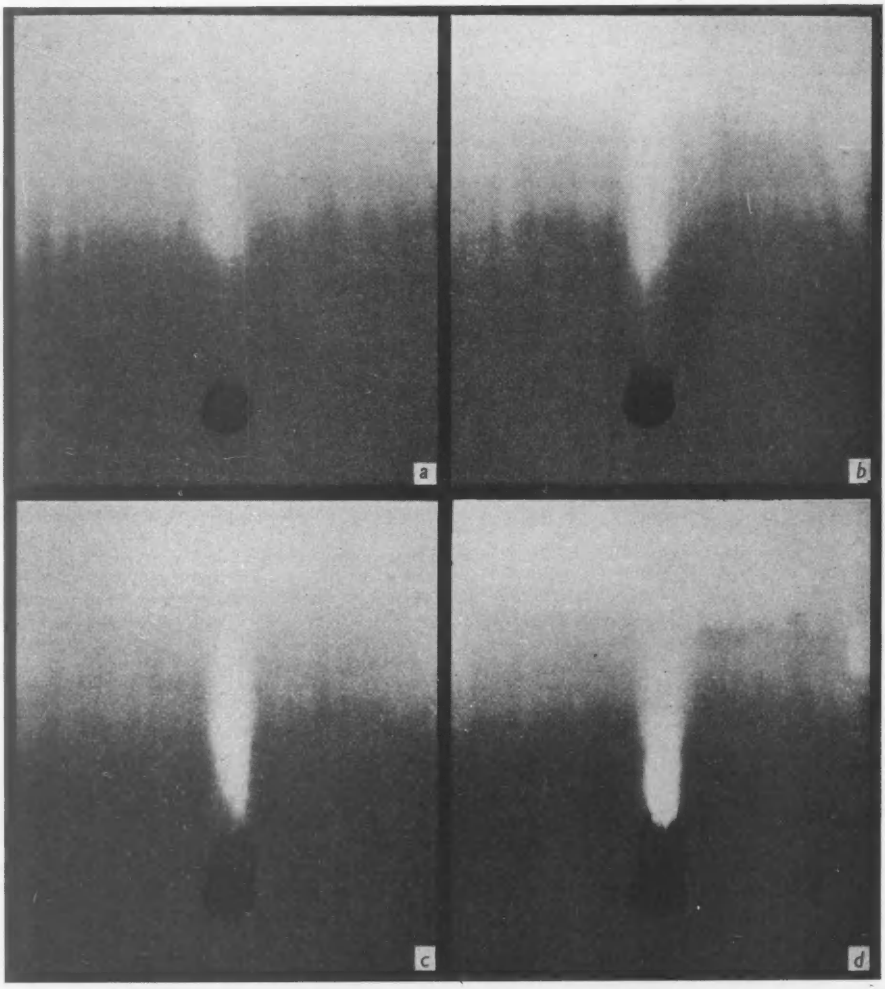

Fig. 1. Paper chromatograms obtained by retention analysis of 5 μl. spots of (*a*) 0·2, (*b*) 0·4, (*c*) 0·6 and (*d*) 0·8% (w/v) protamine sulphate solution with 0·1% erythrosine solution.

Attempted estimation of insulin and protamine by retention analysis

Portions (20 μl.) of 1% insulin solution and of 1% protamine sulphate solution were mixed and half of the mixture applied to each of two 10 cm. strips of Whatman no. 1 filter paper, which were then developed with the aqueous butanol-acetic acid mixture. The strips were dried and one was dipped into a 0·1% (w/v) Solway purple solution; from the positions of the two coloured zones thus formed, the positions of the insulin and protamine on the undyed strip were noted.

This assay strip was cut into four portions each about 8 cm. wide, one portion containing the protamine zone, another the insulin zone and the third and fourth portions containing no protein. Measured amounts (10 μl.) of 1% insulin solution and of 1% protamine sulphate solution were applied to the third and fourth portions respectively to serve as standards.

These four portions of the chromatogram were retention-analysed as described above with a 0·1% solution of

erythrosine, this having been found to work better with protamine than did Solway purple.

The following retention areas were obtained: standard (10 μl. of 1% insulin solution) = 123 sq.mm.; test solution (insulin band) = 179 sq.mm.; standard (10 μl. of 1% protamine sulphate solution) = 312 sq.mm.; test solution (protamine band) = 320 sq.mm.

From these results the insulin zone is apparently equivalent to 14·5 μl. of a 1% insulin solution, whereas only 10 μl. of 1% insulin solution had been chromatographed. The protamine zone, on the other hand, gave an area equivalent to 10·2 μl. of 1% protamine sulphate solution, a result in excellent agreement with the theoretical value of 10 μl.

Presumably, therefore, before insulin can be estimated by retention analysis it is essential that a more compact zone should be obtained than is formed when insulin is chromatographed with the solvent mixture described above. In an attempt to obtain a more suitable insulin zone, another chromatogram was prepared as before. The portion containing the protamine zone was cut off and the insulin zones were developed further with dilute acetic acid (1 part glacial acetic acid to 4 parts of water), which had been shown in preliminary experiments to form a compact zone at the solvent front. The resulting zone was retention-analysed as before, with the following results: standard (10 μl. of 1% insulin solution) = 117 sq.mm.; test solution (insulin band) = 151 sq.mm. Again the result was higher than anticipated, the area being equivalent to 12·9 μl. instead of 10 μl. of 1% insulin solution.

Estimation of protamine in protamine zinc insulin

As the retention analysis of insulin after separation from protamine by chromatography gave unsatisfactory results, attention was concentrated on the estimation of protamine, since this remained in the form of a compact spot readily amenable to retention analysis. Various samples of protamine zinc insulin were freed from insulin by paper chromatography and the residual protamine spot was assayed by retention analysis. Portions of the precipitates and supernatant liquors from these samples were also examined in the same way in order to obtain an estimate of the distribution of the protamine between the precipitates and the supernatant solutions. This was done by centrifuging portions of the protamine zinc insulin sample under investigation, separating the supernatant liquor from the precipitate, dissolving the latter in a few drops of dilute HCl and diluting the solution thus obtained to the original volume with distilled water. Portions (50 μl.) of the original protamine zinc insulin suspension, the supernatant liquor, and the dissolved precipitate were chromatographed and the protamine-containing portions of the filter papers were examined by retention analysis as described above. The protamine content was calculated from the retention areas by reference to a standard curve prepared by treating in a similar manner standard solutions of protamine sulphate. The results are shown in Table 2.

Table 2. *Protamine and insulin contents of various samples of protamine zinc insulin*

(For analytical procedures see text. Figures in table are mg./100 ml. Values given are mean and range. Figures in brackets represent number of assays done.)

Protamine zinc insulin sample no.	Insulin			Protamine		
	Total	Precipitate	Supernatant	Total	Precipitate	Supernatant
AB836 (40 units)	162± 6 (5)	162±30 (4)	Too low to measure	30± 9 (6)	34± 7 (2)	Too low to measure (2)
C208165 (40 units)	204± 3 (2)	250 (1)	Too low to measure	50±10 (6)	51± 6 (3)	5±5 (3)
S919 (40 units)	170± 7 (3)	133± 3 (2)	Too low to measure	36±10 (6)	39± 6 (3)	4±4 (4)
SD342442 (40 units)	160±13 (2)	151 (1)	Too low to measure	41±11 (5)	31±10 (2)	Too low to measure (1)
L4134 (40 units)	158± 8 (2)	178± 1 (2)	Too low to measure	42± 8 (5)	46± 3 (2)	3±3 (2)
O7510 (40 units)	—	—	—	8± 2 (5)	4± 4 (3)	7±6 (3)
B119 (40 units)	150±34 (5)	134±10 (3)	Too low to measure	43± 4 (6)	44± 0 (2)	Too low to measure (1)
B120 (40 units)	155±18 (7)	156± 1 (2)	Too low to measure	48± 9 (5)	38± 7 (4)	Too low to measure (1)
B121 (40 units)	187±35 (5)	150±19 (3)	Too low to measure	51± 6 (6)	42± 1 (2)	Too low to measure (1)
B122 (80 units)	335±31 (3)	338±25 (3)	Too low to measure	96± 8 (6)	86± 7 (2)	Too low to measure (2)

Estimation of insulin in protamine zinc insulin

The amount of insulin present in the volume of protamine zinc insulin suspension adequate for the estimation of protamine was found to be insufficient for the estimation of insulin by Dickinson's method. Accordingly, a second chromatographic separation was carried out on a rectangular sheet of Whatman no. 1 filter paper. Up to 400 μl. of the solution were streaked in a broad band across one end of the paper and about 6 cm. from the edge. The chromatogram was then developed with the upper phase obtained by equilibrating a mixture of *n*-butanol (75%) and acetic acid (25%) with water. After drying, the paper was soaked

in a 0·02% (w/v) aqueous solution of bromocresol green for 5 min. This resulted in adsorption of the dye by both the protamine band and the insulin band. The background colour was removed by washing the strips for 20 min. in three changes of 2% (w/v) acetic acid and the strips were then dried. Fig. 2 illustrates the appearance of a typical chromatogram after exposure to ammonia vapour to intensify the colour. A section 6 cm. wide containing the insulin band was cut out and supported vertically with the upper end bent over and dipping into a borate buffer solution of pH 9·2. This was prepared by mixing 50 ml. of 0·1 M-boric acid in 0·2 M-KCl solution with 26·7 ml. of 0·2 N-NaOH solution and diluting to 200 ml. This eluted the insulin-dye complex, and the eluate, which was collected at the lower end of the strip, was made up to 10 ml.

A 50 μl. spot of the solution was applied to a rectangular sheet of filter paper (10 × 30 cm.) 2 cm. from the longer and 8 cm. from the shorter edge. The latter was bent over so that it dipped 3 cm. into the trough of the chromatographic apparatus containing the upper phase of the *n*-butanol-glacial acetic acid-water mixture described above. After development in a closed vessel for about 8 hr., during which time the solvent front travelled the whole length of the paper and the insulin spot moved about 15 cm., the filter paper was dried in a current of warm air. A 3 cm. strip was then cut from the end of the paper that had been immersed in the solvent, and discarded. The remaining sheet was cut into two portions 10 cm. from the new edge thus formed, and the smaller of the two pieces, containing the protamine spot 2 cm. from one edge, was supported with this edge

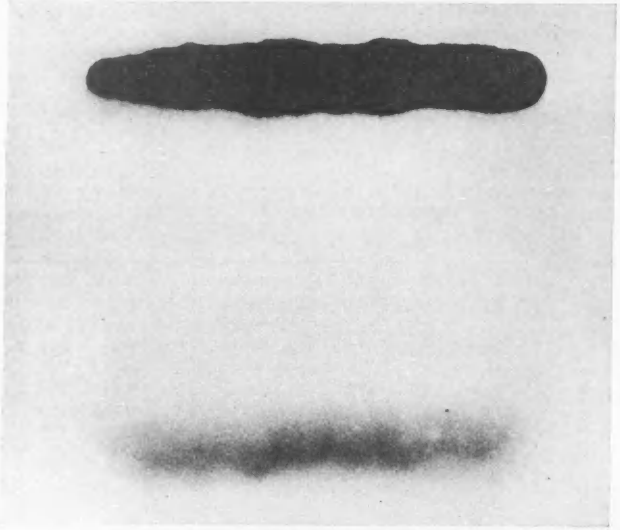

Fig. 2. Paper chromatogram showing bands of protamine (above) and insulin (below) obtained by developing protamine zinc insulin solution with aqueous butanol-acetic acid and staining with bromocresol green solution.

A 6 cm. portion containing no protein band was cut out from the paper and eluted in the same way for use as a blank. The optical density of the eluate was measured against the blank solution in a Spekker absorptiometer using Ilford filter no. 607.

Portions of insulin solutions of various concentrations were chromatographed and the insulin bands treated as described above. A graph was plotted connecting the insulin concentration with the absorptiometer reading. The relation was found to be linear between 0·25 and 1·5% of insulin. The insulin content of the unknown solution was calculated from this standard curve. The results are shown in Table 2.

Recommended method for the estimation of protamine and insulin in protamine zinc insulin

The procedure finally adopted for the estimation of protamine and insulin in protamine zinc insulin suspension was as follows:

dipping into a 0·1% solution of erythrosine and the dye allowed to rise about 5 cm. above the spot. The area of the white V-shaped wedge was then measured by tracing on to graph paper and the results calculated from a standard curve prepared by treating known amounts of protamine in the same manner.

To estimate the insulin content a fresh chromatogram was prepared by streaking 200–400 μl. of protamine zinc insulin solution about 6 cm. from and parallel to the shorter edge of a rectangular sheet of filter paper similar to that used in the protamine estimation. The chromatogram was developed and dried as before. The paper was dipped into a 0·02% solution of bromocresol green for 5 min. and then washed for 20 min. in three changes of 2% acetic acid to remove the background colour. The paper was again dried, the portion containing the insulin zone was cut out and the insulin-dye complex eluted with a borate buffer solution (pH 9·2) and subjected to absorptiometry exactly as described above.

SUMMARY

1. Insulin can be separated from protamine by paper chromatography developing with the upper phase of a mixture of *n*-butanol, glacial acetic acid and water (3:1:4 by vol.). A dye such as bromocresol green is used for locating the zones.

2. The concentration of protamine and of various proteins in a solution can be estimated on filter paper by retention analysis with a suitable dye, e.g. erythrosine. The area of the unstained wedge formed above the protein spot is proportional to the amount of protein present. The method can be used to estimate the amount of protamine in protamine zinc insulin after removal of insulin by paper chromatography.

3. The concentration of insulin in protamine zinc insulin can be estimated, after separating the insulin from protamine by paper chromatography, by staining with bromocresol green solution, eluting the insulin-dye complex and comparing the colour of the eluate with that given by a standard solution of insulin treated in the same way.

4. Data are recorded on the composition of a number of suspensions of protamine zinc insulin and on the distribution of the components between the phases of the suspension.

We thank the Directors of Allen and Hanburys Ltd. and The British Drug Houses Ltd. for permission to publish this paper.

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Studies on Cholinesterase

7. DETERMINATION OF THE MOLAR CONCENTRATION OF PSEUDO-CHOLINESTERASE IN SERUM

By D. K. MYERS

Pharmaco-therapeutic Laboratory, University of Amsterdam, Amsterdam, Holland

(*Received* 17 *October* 1951)

The classical theory of reversible enzyme-substrate and enzyme-inhibitor reactions postulates that enzyme and substrate, or enzyme and inhibitor, form a reversible combination according to mass law principles (Michaelis & Menten, 1913). The mechanism of enzyme-inhibitor reactions has been analysed from this theoretical standpoint by Straus & Goldstein (1943) and Goldstein (1944), with special consideration being given to the concentration of free enzyme and free inhibitor. They pointed out that the degree of inhibition of enzyme activity effected by a certain concentration of inhibitor will be dependent in part upon the enzyme concentration when the so-called 'dissociation' or Michaelis constant of the enzyme-inhibitor system is of the same order of magnitude or much smaller than the molar enzyme concentration employed.

The theoretical treatments given by Goldstein (1944) were illustrated with experimental data obtained, using a pseudo-cholinesterase preparation as the source of enzyme and eserine as the enzyme inhibitor. In this system it is possible to obtain appreciable inhibition of the cholinesterase activity with eserine concentrations as low as 10^{-8} M; nevertheless, the degree of inhibition is practically independent of enzyme concentration under the experimental conditions. Thus it was not possible to confirm the theoretical formulations completely at that time, or to arrive at any definite value for enzyme concentration in the cholinesterase system.

Since that time, other investigators, notably Bain (1949), have attempted to determine cholinesterase concentration by the use of irreversible alkylphosphate inhibitors such as di*iso*propyl fluorophosphonate (DFP) and tetraethyl pyrophosphate (TEPP). The lack of theoretical criteria which could be applied to the results with these inhibitors makes this a rather unreliable method for determining cholinesterase concentration. Moreover, the results obtained do not correspond with those expected of

an irreversible inhibitor which combines specifically and only with the active centre of the cholinesterase on an equimolar basis.

However, it has been observed that the reversible inhibition of pseudo-cholinesterase by the very potent inhibitor, the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide (Nu 683), is dependent upon the cholinesterase concentration (Myers, 1950) and that the experimental results obtained correspond with the theoretical predictions of Straus & Goldstein (1943) and Goldstein (1944) for a competitive reversible inhibitor of high potency. Evidently the concentrations of Nu 683 which produce appreciable inhibition of pseudo-cholinesterase activity are of the same order of magnitude as the concentrations of pseudo-cholinesterase. By applying the theoretical formulations given by Goldstein, pseudo-cholinesterase concentration in serum has been calculated by a method which appears to be completely justified theoretically.

METHODS

The activity of the cholinesterases was measured manometrically by a method similar to those described previously (Mendel & Rudney, 1943; Hawkins & Mendel, 1949; Myers, 1950). The medium used was a 0·025 M-bicarbonate solution saturated with 5% CO_2 + 95% N_2 (v/v), pH 7·4 at 37·5°. Dialysed human, horse, dog or rat serum was used as source of pseudo-cholinesterase, usually with 0·006 M-benzoylcholine as substrate (Mendel, Mundell & Rudney, 1943). Esterase activity for a wide range of cholinesterase concentrations was kept within accurately measurable range by varying the total volume of solution between 2·0 and 5·0 ml. and by the use of 0·06 M-acetylcholine at very low enzyme concentrations with horse and human serum. The cholinesterase activity was, however, always calculated out to the value it would have had if 5·0 ml. of the same solution had been used and if benzoyl choline had been the substrate.

The pseudo-cholinesterase inhibitor in most of these experiments was Nu 683, described by Hawkins & Gunter (1946). This inhibitor is still not sufficiently potent to allow the calculation of enzyme concentration from inhibition in a system where enzyme, inhibitor and substrate are all equilibrated, as has been recommended by Goldstein (1944). Therefore, the measurements must of necessity be done with a system in which only enzyme and inhibitor are in equilibrium. For a competitive reversible enzyme-inhibitor system of this kind, the relationship between inhibitor concentration I and fractional activity a is expressed as

$$I = K_I \frac{(1-a)}{a} + (1-a)E \quad \text{(Goldstein, 1944).}$$

The concentration of inhibitor required to produce 50% inhibition of the enzyme activity can be designated as I_{50}. When the ratio $E/K_I < 0{\cdot}1$, the concentration I_{50} will be approximately equal to K_I and independent of the enzyme concentration E (zone A). When $E/K_I > 100$, the concentration I_{50} will be directly proportional to E and independent of the Michaelis constant K_I (zone C). For intermediate values of E/K_I, I_{50} will depend upon the values of both E and K_I (zone B) (Straus & Goldstein, 1943; Goldstein, 1944). As will be shown below, the pseudo-cholinesterase-eserine system used by Goldstein (1944) must have been in zone A. The pseudo-cholinesterase-Nu 683 system used in the present investigation usually lies in the lower part of zone B so that the values of both E and K_I can be accurately determined.

The equation above can be applied only when sufficient time has been allowed to ensure complete equilibration of the enzyme-inhibitor system. The time of incubation should not be too long, however, or errors may be introduced by the slow enzymic hydrolysis of the carbamic acid ester linkage in the combined inhibitor. Another factor is the progressive displacement of combined inhibitor and consequent increase in the fractional activity caused by the addition of substrate to the equilibrated enzyme-inhibitor system. The importance of these three factors has been stressed by Goldstein (1944), but, as will be described below, the possible errors which they would introduce are relatively small in the case of the enzyme-inhibitor system with Nu 683 and the pseudo-cholinesterase of human or horse serum. During the course of these investigations, preliminary experiments were done with the *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu 1250) (Hawkins & Mendel, 1949) as inhibitor of the true cholinesterase activity of purified human erythrocytes (Mendel & Rudney, 1943). In this case the rate of displacement of combined inhibitor by added substrate proved to be appreciable under the experimental conditions. In addition, the ratio E/K_I was still quite low so that the shift in I_{50} was relatively small. Both of these factors increase the possibility of error in the estimations of enzyme concentration and consequently this enzyme-inhibitor system did not seem suitable for an accurate determination of cholinesterase concentration.

For purposes of greater accuracy with Nu 683, the value of I_{50} at each enzyme concentration was estimated from a series of determinations with different inhibitor concentrations covering the range from 10 to 90% inhibition of the pseudo-cholinesterase activity. The precise value of I_{50} was obtained by plotting the fractional activity a against pI ($-\log I$) for each set of results, as recommended by Goldstein (1944). The theoretical equation relating inhibitor to enzyme concentration then becomes $I_{50} = K_I + 0{\cdot}5E$. If I_{50} is determined for a series of different enzyme concentrations, a plot of I_{50} against the relative concentration of enzyme should give a straight line with intercept equal to K_I and a slope equal to one-half the molar concentration of enzyme.

RESULTS

The most convenient system for accurate determination of pseudo-cholinesterase concentration was that with Nu 683 as inhibitor and the pseudo-cholinesterase of dialysed human serum as enzyme. It had already been shown (Myers, 1950) that this enzyme-inhibitor system exhibits the predicted characteristics (Goldstein, 1944) of a competitive reversible system for which the ratio of E/K_I has a value appreciably greater than 0·1 and in which the inhibitor combines specifically with the enzyme active centre on an equimolar basis ($n = 1$). This is further illustrated by Fig. 1, in which typical

experimental points are compared with the theoretical curves expected for an enzyme-inhibitor system in zone B ($0{\cdot}1 < E/K_I < 100$) (see a later section for theoretical treatment).

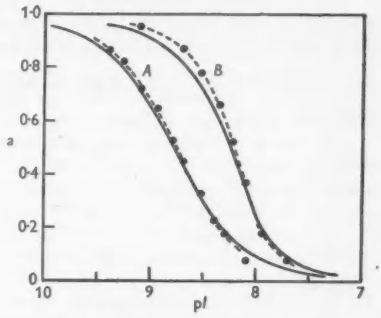

Fig. 1. Comparison of the theoretical curves (———) expected for a competitive reversible inhibitor in zone B with the experimental curves (------) obtained using Nu 683 as inhibitor of the pseudo-cholinesterase activity of dialysed human serum. Theoretical curves calculated for $K_I = 8{\cdot}8 \times 10^{-10}$ M-Nu 683 and $E = 2{\cdot}3 \times 10^{-9}$ M-pseudo-cholinesterase/100 μl. CO_2 activity. Experimental points of curve A obtained after 50 min. incubation of the Nu 683 with a 2% (v/v) serum solution giving 70 μl. CO_2/5 ml. solution/20 min.; those of curve B with a 12% (v/v) serum solution giving 448 μl. CO_2/5 ml. solution/20 min. pI represents the negative log. of the molar concentration of Nu 683 and a the fractional activity in the presence of this inhibitor.

Table 1. *Effect of added benzoylcholine in displacing Nu 683 combined with the pseudo-cholinesterase of human serum*

(5 ml. of 6% (v/v) dialysed human serum in 0·025 M-bicarbonate was incubated with 6×10^{-9} M-Nu 683 for 50 min. at 37·5° before adding 0·006 M-benzoylcholine at time = 0.)

Time after addition of benzoylcholine (min.)	Activity, for time stated, of control serum (μl. CO_2)	Activity, for time stated, of serum incubated with Nu 683 (μl. CO_2)	Fractional inhibition by Nu 683 ($1-a$)
2–7	49·5	12·0	0·758
7–12	55·5	13·0	0·766
12–17	52·5	14·5	0·724
17–22	55·5	15·0	0·730
Average 2–22 min.			0·745
22–32	112·0	32·5	0·710
32–42	114·5	36·5	0·681
42–52	110·5	38·5	0·651
52–62	112·0	43·0	0·616
62–72	111·5	47·5	0·574
72–82	112·5	50·0	0·556
82–92	114·0	55·0	0·517

The error introduced by the displacement of combined inhibitor after addition of substrate was relatively small when the activity was measured over the period 2–22 min. after the addition of benzoylcholine (Table 1). However, the fact that Nu 683 combined with pseudo-cholinesterase was displaced by added benzoylcholine does indicate that Nu 683 is a reversible competitive inhibitor of pseudo-cholinesterase.

It was also necessary, as noted previously, to determine an optimal time of incubation of the enzyme with inhibitor, that is, a time which would be long enough to ensure relatively complete equilibration of the enzyme-inhibitor, but not so long as to allow any appreciable destruction of inhibitor to take place. To this end, a series of determinations of pI_{50} ($-\log I_{50}$) after various times of incubation was carried out using 2 and 12% serum concentrations. The results obtained are given in Table 2; from this data, a standard time of 50 min. incubation was arbitrarily selected for use in subsequent determinations.

Table 2. *Changes in* pI_{50} *after incubation of 2 and 12% (v/v) solutions of human serum with Nu 683 for various times at 37·5°*

Time of incubation (min.)	pI_{50} 2% serum	pI_{50} 12% serum
30	8·83	8·18
50	8·77	8·21
75	8·79	8·07
160	8·64	7·92
270	8·53	7·62

A series of experimental a–pI curves, similar to those shown in Fig. 1, was determined for a total of fifteen different enzyme concentrations with several samples of serum. From each of these curves a value of I_{50} was calculated. The enzyme activity was directly proportional to the serum concentration with any one sample of serum (Fig. 2), but the activity per unit volume varied from one sample to another, as would be expected. Therefore the relative enzyme concentration was expressed on the basis of enzyme activity. A plot of I_{50} against relative enzyme concentration gave the experimental points of Fig. 3. The best straight line, fitted to these points by the method of least squares, proved to be that represented by the equation $I_{50} \times 10^9 = 0{\cdot}880 + 1{\cdot}15$ (per 100 μl. activity).

From the theoretical equation $I_{50} = K_I + 0{\cdot}5E$, it follows that $K_I = 8{\cdot}8 \times 10^{-10}$ M-Nu 683 and

$$E = 2{\cdot}3 \times 10^{-9}\text{ M-pseudo-cholinesterase}$$

in a solution of which 5 ml. gave an activity of 100 μl. CO_2/20 min. with 0·006 M-benzoylcholine as substrate, when measured under the standard conditions of 37·5°, pH 7·4 and 0·025 M-sodium bicarbonate medium. This same concentration of the pseudo-cholinesterase of human serum would

give an activity of 292 μl. CO_2/20 min. under the same conditions when using 0·06 M-acetylcholine as substrate.

An enzyme activity of this order of magnitude is usually obtained with a concentration of about 3% human serum. For a full serum which gives an activity of 700 μl. CO_2/ml. serum/20 min. with benzoylcholine, the concentration of pseudo-cholinesterase would be precisely 35 times as high as the figure given above. That is to say, the concentration of pseudo-cholinesterase in full human serum would be about 8×10^{-8} M on the average.

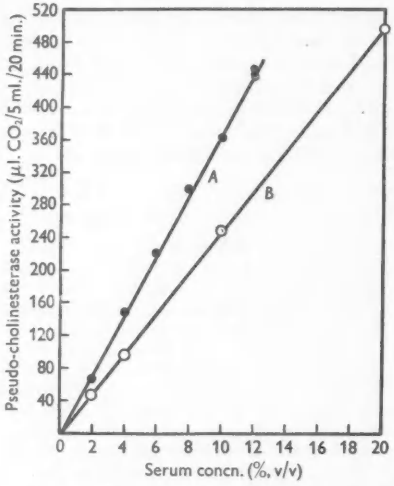

Fig. 2. Linear relationship between the pseudo-cholinesterase activity and the concentration of serum used for two different samples of dialysed human serum. The original activity of the full serum corresponding to line *A* was 820 μl. CO_2/ml./20 min.; this was diluted to 1·12 times the original volume in the process of dialysis. The full serum corresponding to the dialysed sample of line *B* had an activity of 685 μl. CO_2/ml./20 min. and was diluted to 1·38 times the original volume in the process of dialysis. The activity was determined using 0·006 M-benzoylcholine as substrate, a fluid volume of 5 ml. below 10% (v/v) dialysed serum and a fluid volume of 2·5 ml. above the 10% serum concentration, a rate of shaking of 150 × 5 cm. strokes/min., and the other conditions as described in the method.

A similar type of determination on dialysed dog serum gave an equation (from 5 points) $I_{50} \times 10^9 = 3{\cdot}2 + 1{\cdot}35$ (per 100 μl. activity), from which $K_I = 3{\cdot}2 \times 10^{-9}$ M-Nu 683 and $E = 2{\cdot}7 \times 10^{-9}$ M-pseudo-cholinesterase in a solution of which 5 ml. gave an activity of 100 μl. CO_2/20 min. with 0·006 M-benzoylcholine under the standard experimental conditions. In this case the ratio E/K_I would be approximately 0·8 for the pseudo-cholinesterase concentration in a solution which gives the particular esterase activity selected as standard, whereas in the case of human serum previously the two values obtained would give a ratio $E/K_I = 2{\cdot}6$. From the theoretical treatments of Straus & Goldstein (1943) and Goldstein (1944), it would be expected that the enzyme-inhibitor system is much

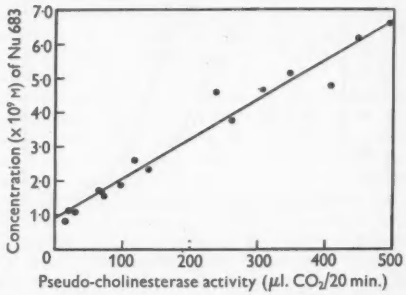

Fig. 3. Relationship between the relative concentration of pseudo-cholinesterase and the molar concentration of Nu 683 which would cause 50% inhibition of the pseudo-cholinesterase activity of dialysed human serum towards 0·006 M-benzoylcholine.

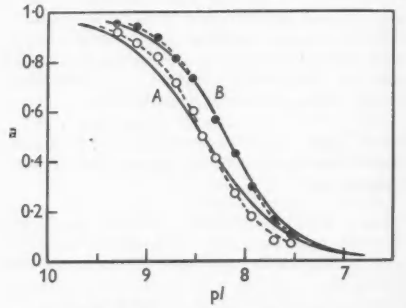

Fig. 4. Comparison of the theoretical (———) and experimental (------) curves obtained using Nu 683 as inhibitor of the pseudo-cholinesterase activity of dialysed dog serum. (cf. Fig. 1). Experimental points of curve *A* obtained after 50 min. incubation of the Nu 683 with a 2% (v/v) serum solution giving 35·4 μl. CO_2/5 ml. solution/20 min.; those of curve *B* with a 12% (v/v) serum solution giving 206 μl. CO_2/5 ml. solution/20 min.

closer to zone *A* with dog serum than with human serum under the conditions used. In agreement with this fact, the *a*–p*I* curves obtained were somewhat flatter and less widely spread than those obtained with human serum. Also the experimental points agree relatively well with the theoretical curves expected from the values of E and K_I obtained (Fig. 4). It is a striking fact that these two sets of curves (Figs. 1 and 4) give similar values

for the pseudo-cholinesterase concentration per unit of activity despite their differences in shape and relative position.

The same concentration of dog serum gave an activity of 186 μl. CO_2/20 min. with 0·06 M-acetylcholine as substrate. From this it can be calculated that the concentration of pseudo-cholinesterase in the 4·54 % dog serum used by Goldstein (63·1 μl. CO_2/20 min. with a volume of 2·2 ml. and with 0·08 M-acetylcholine as substrate) was probably $2{\cdot}07 \times 10^{-9}$ M, as compared with the value $E < 1{\cdot}8 \times 10^{-8}$ M used by Goldstein (1944). This would mean that the ratio E/K_I with eserine as inhibitor ($K_I = 3{\cdot}11 \times 10^{-8}$ M) was of the order of 0·067 in Goldstein's investigations, and justifies the application of zone *A* equations to the experimental data obtained by Goldstein (1944) with eserine as the pseudo-cholinesterase inhibitor.

pseudo-cholinesterase in the serum. In human serum or horse serum, however, the amounts of true cholinesterase present are so small that no appreciable error will be introduced by the use of acetylcholine as substrate for the pseudo-cholinesterase.

Similar determinations of the values of E and K_I were also carried out with the pseudo-cholinesterases of horse, rat and mouse sera. The relative activities of the pseudo-cholinesterases of rat and mouse serum towards acetylcholine were determined by the use of Nu 1250 and half-saturated ammonium sulphate as previously described for dog serum. The results obtained are summarized in Table 3.

Difficulties were encountered with rat serum in that the relatively low pseudo-cholinesterase activity of the full serum does not permit such a wide range of enzyme concentrations to be used in

Table 3. *Comparison of the values of K_I and E as determined with Nu 683 for the pseudo-cholinesterases from the sera of different species*

Source of serum	Activity of full serum towards benzoylcholine (μl. CO_2/ml./ 20 min.)	No. of determinations of I_{50}	K_I ($\times 10^{-9}$ M) Nu 683	E_{BCh}* ($\times 10^{-9}$ M)	ACh/BCh†	E_{ACh}* ($\times 10^{-10}$ M)
Mouse	355	7	0·56	3·27	8·2	3·98
Rat	180	8	3·3	2·05	4·25	4·82
Horse	790	7	0·72	2·24	3·35	6·70
Man	685–820	15	0·88	2·30	2·92	7·89
Dog	340	5	3·2	2·70	1·62	16·7

* E_{BCh} represents the molar concentration of pseudo-cholinesterase in a solution of which 5 ml. give an activity of 100 μl./CO_2/20 min. with 0·006 M-benzoylcholine as substrate under the standard experimental conditions used. E_{ACh} defined similarly but with 0·06 M-acetylcholine as substrate.

† ACh/BCh is the ratio of pseudo-cholinesterase activity towards 0·06 M-acetylcholine over that towards 0·006 M-benzoylcholine.

However, it should be noted that dog serum usually contains appreciable amounts of true cholinesterase as well as the pseudo-cholinesterase, and that both of these enzymes are capable of hydrolysing acetylcholine even at high concentrations of this substrate (Mendel & Rudney, 1943; Mendel *et al.* 1943).

To determine the relative pseudo-cholinesterase activity towards acetylcholine and benzoylcholine, it was first necessary to eliminate all true cholinesterase activity towards acetylcholine. This was done by two methods, by selectively inhibiting the true cholinesterase activity with 10^{-7} M-Nu 1250 (Hawkins & Mendel, 1949), and by precipitating the true cholinesterase from the serum by addition of an equal volume of saturated ammonium sulphate to the serum (Hawkins & Mendel, 1949). The residual pseudo-cholinesterase activity towards acetylcholine and benzoylcholine was subsequently determined in both cases. With the above example of dog serum, it appeared that only 162 of the 186 μl. CO_2/20 min. with 0·06 M-acetylcholine as substrate were actually due to the activity of the

these determinations as in the other cases. It is known that female rat serum contains much larger amounts of pseudo-cholinesterase than the serum of male rats (Mundell, 1944); the same is also true to a lesser extent for the sera of male and female mice. Therefore, pooled samples of female rat serum and of female mouse serum were employed for these determinations. The pseudo-cholinesterase activity of the female rat serum was 180 μl. CO_2/ml. serum/ 20 min. with benzoylcholine as substrate; this is relatively high for rat serum, but even so the activity is still somewhat lower than that of the other types of sera used. In addition, the ratio E/K_I was relatively low so that the values of E and K_I could not be determined as accurately as with human serum or horse serum.

DISCUSSION

The absolute activity of pseudo-cholinesterases

The results given in Table 3 show that the pseudo-cholinesterases from mouse, rat, horse, human and dog serum form a series in which the ratio of pseudo-

cholinesterase activity towards 0·06 M-acetylcholine over that towards 0·006 M-benzoylcholine becomes progressively smaller. The values range from 8·2 to 1·6, a difference of 5·1-fold. In this same series, the pseudo-cholinesterase concentration which gives one unit of activity towards acetylcholine becomes progressively larger, that is to say, the absolute activities of the pseudo-cholinesterases in this series becomes progressively smaller. The values obtained cover a range of 4·2-fold. This is a surprising finding, but closer examination of the results show that there is not a close correlation between the figures in these two series. Moreover, the absolute activity of the pseudo-cholinesterases towards benzoylcholine do not form a parallel series. Thus it seems doubtful that there might be some theoretical significance for the rough correlation in this series of pseudo-cholinesterases between the absolute activity towards acetylcholine and the ratio of activity towards acetylcholine over that towards benzoylcholine.

The Michaelis constant for Nu 683 also varies considerably from one pseudo-cholinesterase to the other, but varies independently of the other characteristics determined. Measurements of the Michaelis constant for acetylcholine show a similar lack of correlation with any of the other results.

From Table 3 it is apparent that the absolute activity of the pseudo-cholinesterases towards benzoylcholine does not vary as much from one type of serum to another as does the absolute activity towards acetylcholine. The former varies by only 1·50 times for the five preparations of pseudo-cholinesterase investigated, the latter by 4·20 times.

The absolute activity, however, is inversely proportional to the pseudo-cholinesterase concentration which gives one unit of activity, regardless of the particular units used. Easson & Stedman (1936) have defined the term absolute activity in terms of a turnover number, i.e. the number of molecules of substrate hydrolysed by one molecule of pseudo-cholinesterase per unit of time. Unfortunately the values given by these authors are not strictly comparable with the above. However, for purposes of comparison with results on other enzymes, turnover numbers can be calculated from the above results on the basis that

$$\begin{aligned} 100\ \mu\text{l. } CO_2/5\text{ ml. soln.}/20\text{ min.} &= 1\ \mu\text{l. } CO_2/\text{ml. soln.}/\text{min.} \\ &= 1\text{ ml. } CO_2/\text{l. soln.}/\text{min.} \\ &= 4{\cdot}46 \times 10^{-5}\text{ moles/l. soln.}/\text{min.} \end{aligned}$$

If this latter figure is divided by any one of the enzyme concentrations given in Table 3 the corresponding turnover number will be obtained, expressed in terms of min.$^{-1}$. The enzyme concentrations are expressed simply in moles of enzyme per litre, but inherent in this method of determining enzyme concentration is the assumption that these concentrations are equivalent to the molar concentration of enzyme active centres. For the present it can only be assumed, in most cases, that each active centre corresponds to one molecule of the enzyme protein.

Thus, for example, each active centre of the pseudo-cholinesterase of horse serum will hydrolyse $6{\cdot}67 \times 10^4$ mol. of acetylcholine or $1{\cdot}99 \times 10^4$ mol. of benzoylcholine per min. under the experimental conditions used. (Compare the value $9{\cdot}35 \times 10^4$ mol. of acetylcholine per min. reported by Berry (1951) after the completion of this paper.) With propionylcholine or butyrylcholine as substrate, this value would be considerably higher. As judged from the results of Augustinsson & Nachmansohn (1949) and Sturge & Whittaker (1950), this turnover number might approach a value of $1{\cdot}4 \times 10^5$ mol. of butyrylcholine per min. A turnover number expressed in this way will be identical with the destruction constant k_D of Goldstein (1944) when the appropriate small correction is made for the effect of substrate concentration.

Another point of some interest is the order of magnitude of the activity of pure pseudo-cholinesterase. The exact molecular weight of pseudo-cholinesterase is unknown, but for a serum albumin we might assume a molecular weight of 70 000 (Kekwick, 1938). Further, assuming that one active centre corresponds to 1 mol. of pseudo-cholinesterase protein, it could be calculated that pure pseudo-cholinesterase from horse serum would have a $Q_{\text{acetylcholine}}$ value of 1 300 000 μl. CO_2/mg. dry wt./hr. This is of a similar order of magnitude to the $Q_{\text{acetylcholine}}$ values of 400 000 and 600 000 for the purest preparations of pseudo-cholinesterase obtained by Strelitz (1944) and Mendel & Mundell (1943) respectively.

The data presented in Table 3 show that the pseudo-cholinesterases from the sera of different species of animal may have considerably different affinities for the same inhibitor. Moreover, the absolute rate of hydrolysis (per mole of enzyme) of the same substrate may also differ from one preparation of pseudo-cholinesterase to another. The magnitude of these variations in the absolute rate of hydrolysis by various pseudo-cholinesterase preparations also depends upon the substrate concerned. This fact has already been indicated by the results of other investigators, which show that the pseudo-cholinesterase from horse serum differs from the pseudo-cholinesterase of human serum in the relative rate of hydrolysis of non-choline esters (Blaschko & Holton, 1949; Adams & Whittaker, 1949; Sturge & Whittaker, 1950).

It has been pointed out by Mendel and co-workers (Mendel & Rudney, 1943; Mendel *et al.* 1943; Mendel & Mundell, 1943; Strelitz, 1944; Hawkins & Gunter, 1946; Hawkins & Mendel, 1949) that pseudo-

cholinesterase is a distinct enzyme, with the same general and characteristic specificity towards certain substrates and certain inhibitors in all cases investigated, regardless of the source of the enzyme. The results of later investigations emphasize the fact that preparations of pseudo-cholinesterase from different sources often show minor differences in the relative specificity for various substrates and inhibitors. Consequently, it has been proposed that the pseudo-cholinesterases from various sources should be regarded as a special group or 'family' of related enzymes rather than as a special enzyme, and that the true cholinesterases, similarly, should be regarded as another special group of closely related enzymes (Bodansky, 1946; Augustinsson, 1948). The results of the present investigation would appear to justify such a concept—to the same extent as it would be justified to consider the haemoglobins, for example, as a group of closely related haem proteins.

In both cases it can be considered that the active centre or active grouping will determine the general characteristic specificity common to all different preparations of the same enzyme, while the minor variations in relative specificity will be determined mainly by the influence of the various species-specific (and possibly organ-specific) protein carriers. Thus it will depend upon the purposes of the investigation in hand whether the pseudo-cholinesterase (or the true cholinesterase, or the haemoglobin) from different sources is to be regarded as a special enzyme (from the viewpoint of physiological function) or as a special group of enzymes (from the viewpoint of enzymic or immunological investigations). The pseudo-cholinesterases from different sources would appear to have the same general characteristics in all cases, but it must be emphasized that the Michaelis constants, reaction velocity constants and enzyme concentration will not be identical from one preparation of pseudo-cholinesterase to another.

The use of inhibitors in the study of pseudo-cholinesterases

It has been found previously that the inhibition of pseudo-cholinesterase by Nu 683 is reversed only very slowly by dialysis or dilution of the enzyme-inhibitor system (Hawkins & Gunter, 1946). Nevertheless, Nu 683 must be a reversible competitive inhibitor of pseudo-cholinesterase since the combined inhibitor is displaced by added substrate. The previous findings are probably to be explained by the very high affinity of Nu 683 for pseudo-cholinesterase and by the fact that the enzyme-inhibitor system lies in zone *B* under the experimental conditions.

The experimental results with this inhibitor show sufficiently good agreement with the theoretical predictions of Straus & Goldstein (1943) and Goldstein (1944) to provide experimental confirmation of the theoretical treatments of these authors, in so far as the concept of three zones of inhibitor action is concerned. And conversely, it would also indicate that Nu 683 combines specifically with the pseudo-cholinesterase active centre on an equimolar basis. Thus the results with this inhibitor should give an accurate determination of pseudo-cholinesterase concentration with the mathematical methods used.

On the other hand, the use of irreversible inhibitors such as DFP or TEPP can lead to erroneous values for enzyme concentration. In the first place it might be noted that the data of Bain (1949) indicate apparent enzyme concentrations which differ by 250 times when these two compounds were used as 'specific' irreversible inhibitors of the true cholinesterase activity of rat brain. Evidently, at least one of the two cannot be a specific cholinesterase inhibitor, but there is no theoretical criterion which could be applied to such results to indicate when an irreversible inhibitor does combine specifically with the cholinesterase active centre on an equimolar basis.

The discrepancies observed in the results with these alkyl-phosphate inhibitors are further illustrated by the data obtained using DFP as an inhibitor of true cholinesterase activity (Fig. 5). The results obtained in this case are more typical of a reversible competitive inhibitor in zone *B* than of a specific irreversible inhibitor, despite the fact that for most practical purposes the inhibition of cholinesterase activity by DFP is completely irreversible under these experimental conditions. But while the experimental results with DFP would indicate an apparent enzyme concentration of $2{\cdot}9 \times 10^{-8}$ moles/100 μl. activity with 0·03 M-acetyl-β-methylcholine under standard conditions, the results obtained with Nu 1250 showed that the true enzyme concentration was no greater than $6{\cdot}3 \times 10^{-10}$ M in the same solution. The reactions of cholinesterases with DFP and similar inhibitors will be dealt with in more detail in a subsequent publication. For the present purposes it is sufficient to note that the explanation of these results with DFP is still uncertain, and that the use of this inhibitor will not give a reliable value for the concentration of cholinesterase under the above conditions.

At least three other groups of investigators (Easson & Stedman, 1936; Ellis, Plachte & Straus, 1943; Straus & Goldstein, 1943) have attempted to measure the pseudo-cholinesterase concentration in horse serum by the use of eserine or prostigmine as inhibitors. Their results have been criticized on theoretical and practical grounds by Goldstein (1944), who himself did not attempt to give anything more than a maximum value for the cholinesterase concentration. The results obtained in the present

investigation with Nu 683 as inhibitor do indicate considerably different concentrations of pseudo-cholinesterase in horse serum from those which have been previously proposed, as would be expected in view of Goldstein's criticism.

The difference between the method used in the present investigation for determination of pseudo-cholinesterase concentration and previous determinations with competitive reversible inhibitors

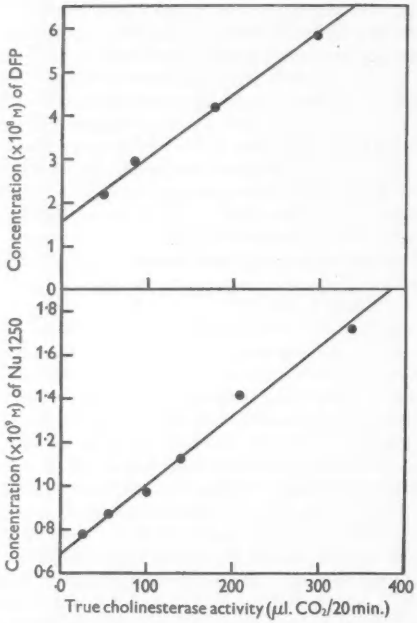

Fig. 5. Relationship between the relative concentration of true cholinesterase and the molar concentrations of Nu 1250 or of DFP which would cause 50% inhibition of the true cholinesterase activity of human erythrocytes towards 0·03 M-acetyl-β-methylcholine. The degree of inhibition effected by various concentrations of Nu 1250 was determined after incubation with the erythrocyte preparation for 80 min., that effected by DFP after incubation for 120 min.

lie in three major points. In the first place, Nu 683 is a much more potent inhibitor of pseudo-cholinesterase than either eserine or prostigmine; consequently the degree of inhibition obtained is more dependent on the enzyme concentration. Secondly, the enzyme concentration and Michaelis constant are determined from the results at several different enzyme concentrations rather than from one set of results as in the generally accepted method of Easson & Stedman (1936). Thirdly, the experimental results are plotted as fractional activity a against pI to give a sigmoid curve. Easson & Stedman (1936), on the other hand, used an equation of the form

$$\frac{I}{(1-a)} = \frac{K_I}{a} + E,$$

and plotted $I/(1-a)$ against $1/a$ on a linear basis. The equation itself is theoretically correct, of course, and this mathematical method of analysing the results would give correct values for E and K_I if the experimental results correspond exactly with the theoretical expectations. But when the data are subject to some experimental error, the use of this equation can give results which are very misleading. Goldstein (1944) has pointed out that this latter method gives a vastly disproportionate weight to the experimental results with very small values of a, and that it is just these results more than any others which are most subject to large experimental errors. The method of plotting a against pI would weight all points to the same extent so that a theoretical curve can be obtained which will give a minimal standard deviation from all of the experimental points (Goldstein, 1944). Thus this method will give a more accurate representation of the experimental data, and the shift in I_{50} concentration with changes in enzyme concentration will allow the calculation of a reliable value for the molar concentration of enzyme. The a–pI plot has a further advantage in that the experimental results can be compared with the theoretical predictions for a competitive reversible inhibitor which combines specifically with the enzyme active centre on an equimolar basis. The combination of all of these factors seems to make this a reliable method for the accurate determination of pseudo-cholinesterase concentration in serum.

SUMMARY

1. The experimental results show that Nu 683 (the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide) is a reversible competitive inhibitor of pseudo-cholinesterase which combines specifically with the pseudo-cholinesterase active centre on an equimolar basis. The ratio of E/K_I has a value appreciably greater than 0·1; this has made it possible to determine pseudo-cholinesterase concentration in serum accurately by a method which appears to be completely justified theoretically. The advantages of this method over those which have been used previously have been discussed.

2. The experimental results obtained with this inhibitor show sufficiently good agreement with the theoretical predictions of Straus & Goldstein (1943) and Goldstein (1944) to provide experimental confirmation of the concept of three zones of inhibitor action.

3. The pseudo-cholinesterase concentrations have been expressed on the basis of enzyme activity under standard conditions. The concentration of pseudo-cholinesterase in the particular samples of full serum used were as follows: horse serum $8{\cdot}8 \times 10^{-8}$ M; human serum, $7{\cdot}9 \times 10^{-8}$ M; female mouse serum, $5{\cdot}8 \times 10^{-8}$ M; dog serum, $4{\cdot}6 \times 10^{-8}$ M; female rat serum, $1{\cdot}7 \times 10^{-8}$ M.

4. The pseudo-cholinesterases from mouse, rat, horse, human and dog serum form a series in which the ratio of pseudo-cholinesterase activity towards 0·06 M-acetylcholine over that towards 0·006 M-benzoylcholine diminishes progressively from 8·2 to 1·6. In this same series, the absolute activity of the pseudo-cholinesterases towards acetylcholine becomes progressively smaller. Other characteristics of the pseudo-cholinesterases also vary considerably from one type of pseudo-cholinesterase to another, but do not show any correlation to the above series.

5. The significance of these results has been discussed with respect to the concept of the pseudo-cholinesterases as a special group of closely related enzymes.

The author is indebted to Dr B. Mendel for his valuable criticisms and suggestions, to Miss M. de Jonge and Miss E. Simons for technical assistance, and to Miss E. F. Purdie for her assistance in preparing this article for publication. Nu 683 and Nu 1250 were obtained through the courtesy of Dr J. A. Aschlimann of Hoffmann-La Roche, Inc., Nutley, N.J., U.S.A., and DFP was kindly supplied by Dr B. C. Saunders.

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The Chemical Estimation of Adrenaline-like Substances in Blood

By H. WEIL-MALHERBE and A. D. BONE
Research Department, Runwell Hospital, Wickford, Essex

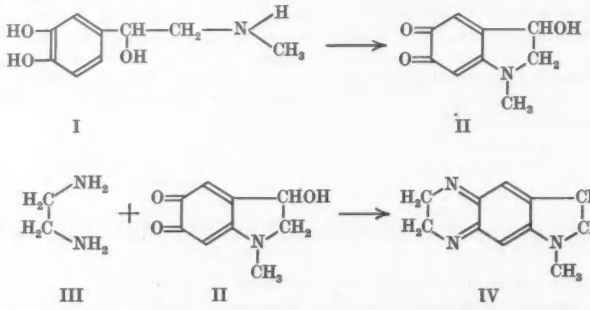
(*Received* 29 *October* 1951)

Of the numerous chemical methods which have been devised for the estimation of adrenaline the fluorimetric techniques seem most likely to reach a degree of sensitivity sufficient for the measurement of concentrations as low as those occurring in peripheral venous blood. Adrenaline, as is well known, forms an unstable fluorescent oxidation product in alkaline solution. Lund (1949*b*) recently described a method in which the fluorescent derivative is stabilized by the addition of ascorbic acid and which is capable of estimating concentrations of not less than 10 μg./l. Recovery of added adrenaline amounted to 80% and a factor was employed to correct for the loss. Lund did not find measurable amounts of adrenaline in venous blood and concluded that adrenaline, if present at all, occurred in concentrations of less than 10 μg./l.

Although the sensitivity of Lund's method could perhaps be increased by suitable modifications, the procedure seems to have other disadvantages. It is stated that adrenaline is quantitatively oxidized to adrenochrome by manganese dioxide without adrenochrome itself being further oxidized. The need for a rapid and complete removal of manganese dioxide is, however, emphasized and variations of such factors as temperature or the activity of the manganese dioxide preparation might, unless carefully controlled, entail the risk of a loss of adreno-

chrome. Secondly, as pointed out by Fischer, Derouaux, Lambot & Lecomte (1950), adrenochrome is liable to be reduced in the presence of ascorbic acid and may thus partly escape transformation to the fluorescent compound in alkaline solution. Whether these side reactions can seriously affect the practical value of the method has not been studied by us.

In the following, a fluorimetric method of adrenaline estimation will be presented which is not, like previous fluorimetric methods, based on the formation of adrenolutine (Lund, 1949*a*), but on a condensation with ethylenediamine. The reaction was discovered by Natelson, Lugovoy & Pincus (1949), but, as far as we are aware, it has not been further studied or applied to blood. The condensation takes place in alkaline solution in which adrenaline is readily autoxidizable. No reaction occurs if oxygen is excluded. If adrenaline is replaced by adrenochrome, a fluorescence intensity curve is obtained, both in presence and in absence of oxygen, which is identical with that produced by equimolar amounts of adrenaline in presence of oxygen. It may therefore be concluded that the condensation is preceded by an oxidation of adrenaline (I) to adrenochrome (II), and the reaction may be formulated as follows:



I II

III II IV

(I) Adrenaline. (II) Adrenochrome. (III) Ethylene diamine. (IV) Condensation product (hypothetical).

The constitution of the condensation product (IV) must at present be regarded as hypothetical.

The fluorescence of the final extract remains constant for at least 24 hr. The fact that the unstable, highly reactive adrenochrome is trapped in the nascent state and quantitatively converted into a completely stable condensation product is the most notable advantage of the method. In addition, the sensitivity is such that adrenaline concentrations of 1 μg./l. can be measured with accuracy; the recovery is quantitative and the use of a correction factor is therefore unnecessary; and finally, strictly additive results are obtained with mixtures of adrenaline and noradrenaline, whereas the production and the decay of fluorescence observed in an alkaline solution of adrenaline is disturbed by noradrenaline (Heller, Setlow & Mylon, 1950).

The procedure of Natelson *et al.* (1949) has been modified: the condensation is carried out by heating the solution at 50° for 20 min. with a mixture of ethylenediamine and an aqueous solution of ethylenediamine dihydrochloride. The free base is added to produce the required degree of alkalinity (pH of the mixture = 10·4) and may be replaced by ammonia. Since the presence of ethylenediamine in the ionized form is essential, it is obviously the reactive agent. For the extraction of the fluorescent condensation product *iso*butanol was used in preference to amyl alcohol; a quantitative extraction was achieved by saturating the aqueous phase with sodium chloride.

Attempts to replace ethylenediamine by *o*-phenylenediamine were abandoned owing to the formation of highly fluorescent oxidation products in the reagent blank.

The initial separation of adrenaline by chromatography introduced by Lund (1949*b*) has been adopted, with some modifications designed to reduce the autoxidation of adrenaline before and during the adsorption. Acid-washed alumina is used and its quantity is reduced. Another point which was found to be particularly important is the removal of traces of heavy metal from the sodium acetate solution that is added to plasma prior to adsorption. This is done by passing the solution through a column of cation-exchange resin. Finally, the precise adjustment of the diluted plasma to pH 8·4 is controlled by a glass electrode.

The blood is drawn directly into a solution of fluoride and thiosulphate which combines antioxidant with anticoagulant properties. The apparent adrenaline content remains constant for at least 24 hr. at refrigerator temperature. In a few instances red blood cells were washed and cytolysed. Their apparent adrenaline content was about equal to that of plasma in confirmation of Lund's results,

but the alumina columns were liable to become clogged when haemolysates were used, and for this reason most estimations were carried out with separated plasma.

METHOD

Reagents

Sodium fluoride-sodium thiosulphate solution. NaF (2 g.) and $Na_2S_2O_3$ (3 g.), are dissolved in glass-distilled water to 100 ml. The solution is autoclaved and transferred to sterile ampoules, each containing about 6 ml.

Sodium carbonate. A 0·5N solution.

Sodium acetate buffered at pH 8·4. A 0·2M solution of sodium acetate is passed over a column of Zeo-Karb 215 (Permutit Co. Ltd.) and adjusted to pH 8·4 by the addition of 0·5N-Na_2CO_3 (about 5 ml./l.) with the aid of a glass electrode. The Zeo-Karb column is prepared by repeated washings with 2N-HCl, water and 4% NaCl, the last being continued until the pH of the filtrate rises to about 6. Excess NaCl is flushed out with glass-distilled water.

Alumina, acid-washed. Al_2O_3 (100 g.; British Drug Houses Ltd., 'for chromatographic adsorption analysis') is stirred with 500 ml. boiling 2N-HCl for 20 min., filtered and washed on the funnel with 500 ml. of hot 2N-HCl. It is then repeatedly washed by decantation with distilled water and dried at 300° for 3 hr.

Acetic acid. A 0·2N solution.

Ethylenediamine dihydrochloride, 2M solution. The salt is prepared from freshly redistilled ethylenediamine by the slow addition of the calculated amount of 6N-HCl (A.R.). Crystals are filtered by suction, dried on a water bath and finally over H_2SO_4.

Ethylenediamine, or ethylenediamine hydrate. Redistilled.

iso Butanol. A laboratory chemical grade is satisfactory.

Procedure

Fluoride-thiosulphate solution (5 ml.) is drawn up into a 20 ml. syringe and about 15 ml. blood collected from the cubital vein. The syringe is emptied into a 25 ml. measuring cylinder and the volume is noted (v_1 ml.). The plasma is separated by centrifugation (v_2 ml.) and an equal volume of sodium acetate buffer added. The mixture is brought to pH 8·40 by the addition of 3–5 drops of Na_2CO_3 solution, precise adjustment being controlled by a glass electrode.

Thistle funnels with a 50 ml. bulb and a 5 mm.-bore stem are used for the chromatographic separation. At a distance of about 18 cm. from the bulb, the stem has a constriction on which is placed a plug of glass wool. The funnel is mounted on a filter flask. The column is prepared by pouring in 0·7 g. of dry acid-washed alumina followed by 5 ml. acetate buffer. With the aid of a drawn-out glass rod the alumina is stirred up until all trapped air bubbles have escaped. After allowing it to settle, mild suction is applied which is so regulated that the rate of filtration does not exceed 20–30 drops/min. The plasma-acetate mixture is added next, followed by 5 ml. acetate buffer and 5 ml. glass-distilled water. The filtrates are rejected. The adsorbed adrenaline is eluted by passing 5 ml. 0·2N-acetic acid, followed by 5 ml. water, through the column.

After the addition of 0·5 ml. ethylenediamine dihydrochloride solution and 0·7 ml. ethylenediamine (or 1 ml. ethylenediamine hydrate) the eluate is heated at 50° for 20 min., cooled to room temperature and saturated with solid NaCl (about 4 g.). The solution is extracted with 6 ml. *iso*butanol in a mechanical shaker for 4 min. and lightly centrifuged to break up the emulsion. The *iso*butanol layer is removed completely and 5 ml. are measured into the fluorimeter tube. To avoid unnecessary transfer the eluate is collected in a glass-stoppered centrifuge tube in which the condensation and extraction procedures are carried out.

A standard containing 0·2 μg. adrenaline in 10 ml. and a reagent blank are carried through the condensation and extraction procedures together with the plasma eluates.

Measurement of fluorescence

A 125W. high-pressure mercury vapour lamp with stabilized arc (Type MBL/D, British Thomson-Houston Co. Ltd.), operated in conjunction with choke and voltage stabilizer (Type MT140A, Advance Components Ltd.) serves as light source. The exciting light is passed through a blue filter (Chance OB10, 2 mm. thick) and focused by a thin-walled, flat-bottomed round flask of 250 ml. capacity, filled with water, on to a thin-walled test tube of non-fluorescent glass (11 mm. internal diameter). The test tube is held inside a light-tight box to which is attached, at right angles to the beam of the exciting light, the photocell compartment of a Unicam S.P. 500 spectrophotometer. The fluorescent light is filtered through a yellow filter (Chance OY4, 2 mm. thick) before entering the photocell. The photocell current is measured with the aid of the electrical controls provided by the Unicam spectrophotometer with slight modifications. It was necessary to change the bias on the first amplifier valve which controls the range of illumination at which the photocell works. This involved fixing a 100000 Ω variable potentiometer between two taps on battery no. 3 of the instrument, and this control was fitted with a built-in switch to return the instrument to its normal operating state when used as a spectrophotometer.

Readings are taken 45 min. after switching on the mercury lamp in order to eliminate drift during the warming-up period. The reagent blank is used to establish the zero position of the 'Transmission' control: by means of the new control for rough adjustment and the 'Dark Current' control for fine adjustment the potential of the photocell at this illumination level is backed-off until balance is obtained with the 'Transmission' dial set at zero. The standard is now introduced into the fluorimeter box, and with the 'Transmission' dial at 100, balance is obtained by operating the 'Sensitivity' control. Next the unknown sample is inserted and balance is established by turning the 'Transmission' control. The position of the dial shows the intensity of the unknown fluorescence as a percentage of the standard fluorescence. When a reading is taken, the shutter which normally protects the photocell is opened and replaced as soon as the necessary adjustments have been made so as to avoid undue fatigue. In each series of readings the position of the zero and the 100 setting is checked at intervals by re-introducing the reagent blank and the standard, respectively. When a series has been completed, the readings are repeated in the same order. Duplicate readings obtained in this way may differ somewhat owing to instabilities in the amplifier circuit or to fluctuations of the mercury lamp output. The mean of the duplicate readings is used to calculate the result.

Calculation

If the fluorimetric measurement indicates the presence of a μg. adrenaline, the amount, per litre of plasma, is

$$\frac{a \times v_1 \times 1000}{v_2(v_1 - 5)} \mu g.$$

RESULTS AND DISCUSSION

Choice of standard

When a standard of 0·2 μg. adrenaline is used to adjust the sensitivity setting the calibration curve obtained is linear (Fig. 1), indicating proportionality between fluorescence intensity and photocell current.

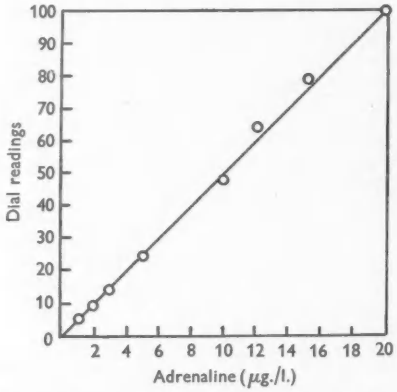


Fig. 1. Calibration curve of fluorimetric estimation of adrenaline.

This setting was optimal for the equipment available. Attempts to increase the sensitivity to the point where a dial reading of 100 was obtained with a standard of 0·1 μg. resulted in a calibration curve which was no longer linear; in addition, there was a disproportionate loss of discrimination. The sensitivity setting obtained with a standard of 0·2 μg. proved satisfactory for our purpose, since with most blood samples the scale readings were between 15 and 20 on a scale of 100 divisions. If it should be required, the sensitivity could no doubt be increased, either by changing the type of photocell, or by intensifying its illumination.

Error of fluorimeter readings

On the scale of 100 divisions, 1 division corresponds to 0·002 μg. adrenaline, given the usual sensitivity setting. Readings were taken to 0·1 of a division, the last figure being obtained by approximate estimation. Owing to slight instabilities in the set-up, duplicate readings differed to some extent. The standard deviation of single readings was calculated from the sum of the squares of the differences (Δ) between n pairs of duplicate readings according to the formula $s = \sqrt{\frac{\Sigma(\Delta^2)}{2n}}$. The results show that the error does not appreciably vary in different ranges of dial readings, the standard deviation being close to 0·5 division throughout (Table 1). This indicates that single readings are accurate to approximately ± 1 division and the mean of duplicate readings to approximately $\pm 1/\sqrt{2} = 0{\cdot}7$ division, or 0·0014 μg. adrenaline.

Table 1. *Error of fluorimeter readings*

No. of samples	Range of dial readings (divisions)	Standard deviation of single reading (divisions)
31	5–10	0·406
67	10–15	0·437
45	15–20	0·388
27	20–40	0·601
15	40–70	0·482

Recovery

Both adrenaline and noradrenaline were quantitatively recovered when pure solutions were carried through the chromatographic adsorption process. The recovery from plasma was studied by adding varying amounts of adrenaline to 10 ml. samples of sterile horse serum. The results of ten such experiments showed that recovery was quantitative with a standard deviation of about 5 % over the entire range investigated (Table 2). To be significant at a probability level of 0·01, the difference between two results must exceed 15 % or, at a probability level of 0·05, 11 %.

Table 2. *Recovery of adrenaline added to horse serum*
(Ten experiments for each quantity of adrenaline added.)

Adrenaline added (μg./10 ml.)	Mean recovery (%)	Standard deviation of recovery
0·02	100·55	6·34
0·03	100·01	4·70
0·05	97·89	4·90
0·10	97·34	4·47

Equally good recoveries, obtained by different methods, have been reported by many other authors. Nevertheless, the estimates of the normal level of blood adrenaline differ widely according to the method used. Obviously, a satisfactory recovery of added adrenaline excludes an underestimate, but not an overestimate. This can only be done by obtaining further evidence.

Specificity

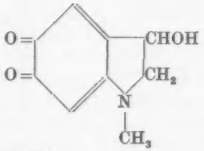
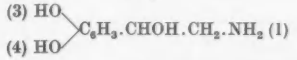
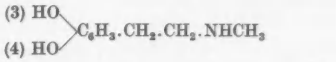
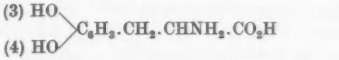
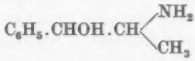
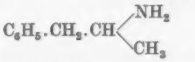
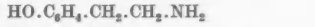
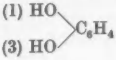
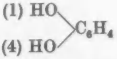
Fluorescence of variable intensity is produced by all catechol derivatives tested, with the exception of dihydroxyphenylalanine (Table 3). This presumably forms a derivative which is not extracted

by *iso*butanol from the alkaline solution, or only to a slight extent. None of the non-catechol substances tested produced any significant fluorescence after their solutions had been carried through the chromatographic adsorption process.

The reaction is thus less specific than that resulting in the formation of adrenolutine or similar compounds, for which indole ring closure is required. But this is of no practical consequence since physiological catechol derivatives which are not capable of indole ring closure, such as dihydroxyphenylalanine and, possibly, dihydroxyphenylserine, are largely eliminated by the extraction procedure.

When the condensation reaction with ethylenediamine was omitted and plasma eluates were directly extracted with *iso*butanol, the fluorescence of the extract was smaller than that of the usual reagent blank. This indicates that none of the fluorescence measured in the usual test is due to preformed plasma components, but is produced entirely during the condensation reaction.

Table 3. *Specificity of method*

	Formula	Relative fluorescence
Catechol derivatives		
L-Adrenaline		100
Adrenochrome		100
L-Noradrenaline		20
Epinine		48
Isoprenaline		56
Catechol		96
L-Dihydroxyphenylalanine		0·2
Other compounds		
Ephedrine		2×10^{-4}
Amphetamine		1×10^{-6}
Tyramine		3×10^{-3}
Resorcinol		~0
Quinol		~0
Nicotinamide		~0

The fluorescence intensity produced by noradrenaline is one-fifth that produced by an equimolar amount of adrenaline. The fluorescence produced by a mixture of both substances is equal to the sum of the fluorescence produced by the separate components. There is thus no mutual interference like that observed under different conditions by Heller *et al.* (1950).

Rate of autoxidation

When plasma was diluted with an equal amount of sodium acetate buffer, adjusted to pH 8·4, and shaken in air, added adrenaline was found to disappear at about the same rate as the preformed material (Table 4). To facilitate autoxidation, sodium thiosulphate was omitted from the anticoagulant solution and the sodium acetate solution used in the experiment was not previously purified by cation exchange.

The experiment shows that in its autoxidizability the preformed substance behaves like adrenaline.

Table 4. *Autoxidation of preformed and added adrenaline in human plasma*

(Blood (150 ml.) collected in 50 ml. 2% NaF (no $Na_2S_2O_3$).)

Adrenaline (μg./l.)			
	Found		
Added	Initial	After 30 min. shaking in air (37°, pH 8·4)	Loss (%)
0	3·1	1·6	48·5
20	22·9	9·8	57

Action of amine oxidase

It could further be shown that an extract of rabbit liver removed preformed material and added adrenaline at an equal rate from plasma (Table 5). The extract was inactivated by boiling and inhibited by ephedrine, a specific inhibitor of amine oxidase (Blaschko, Richter & Schlossmann, 1937). The inhibition by ephedrine was the same whether the enzyme acted on the preformed substance or on added adrenaline. It may therefore be concluded that the action of the liver extract was due to its content of amine oxidase, and that the substance preformed in plasma is an amine derived from catechol with affinities for amine oxidase similar to those of adrenaline.

It remained to be shown that the entire fluorescence measured in the test was due to this substance and was not the resultant of several components. It was found that amine oxidase quantitatively removes the preformed material from plasma (Fig. 2). The plot is that of a first-order reaction, i.e. a straight line is obtained on a semi-logarithmic scale. The bulk of the reacting material is therefore either a single substance or a mixture of substances with similar affinities for the enzyme.

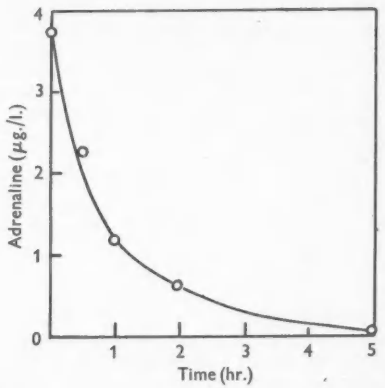


Fig. 2. Action of amine oxidase on preformed reacting material in human plasma. Enzyme prepared as in experiment of Table 5. Plasma (15 ml.) incubated with 3 ml. liver extract at 37°. Results were corrected by subtracting the fluorescence obtained in controls containing 15 ml. water + 3 ml. liver extract.

Table 5. *Action of amine oxidase on preformed and added adrenaline in plasma*

(Enzyme: rabbit liver homogenized with 1 part water and centrifuged. Residue washed with 1 part water. Combined supernatants dialysed against running distilled water at 0° for 16 hr. Blood (150 ml.) collected in 50 ml. NaF-$Na_2S_2O_3$ solution. Samples contained 15 ml. plasma, with or without 3 ml. liver extract. Incubation at 37° for 2 hr.)

		Adrenaline found (μg./l.)	
Sample no.	Addition to plasma	Preformed	After adding 10 μg./l.
S1	0	3·7	11·2
S2	0·01 M-Ephedrine	4·0	11·7
S3	Liver extract, boiled	4·3	13·0
S4	Liver extract + 0·01 M-ephedrine	3·4	10·1
S5	Liver extract	1·4	4·1
Disappearance (S3 − S5)		2·9	8·9
Percentage disappearance $\left(\frac{(S3-S5)}{S3}\times 100\right)$		67·5	68·5
Percentage inhibition by ephedrine $\left(\frac{(S4-S5)}{(S3-S5)}\times 100\right)$		69	67·5

The identity of the adrenaline-like substances in blood

It is now generally accepted that a mixture of adrenaline and noradrenaline, in varying proportions, occurs not only in the adrenal medulla, but in all tissues containing functional post-ganglionic sympathetic fibres, and that it is discharged into the blood stream when these fibres are stimulated (cf. references in a review by von Euler, 1950). The sympathomimetic amine occurring in human and cattle blood is, according to von Euler & Schmiterlöw (1947), largely L-noradrenaline with an admixture of about 4 % adrenaline; rabbit blood, on the other hand, contains mainly adrenaline, according to West (1947). A third sympathomimetic amine, hydroxytyramine, may be the physiological precursor of noradrenaline (cf. Blaschko, 1950). Traces have been found in the adrenals (Goodall, 1950) and in urine (Holtz & Credner, 1942; von Euler & Hellner, 1951), but its concentration in blood is probably negligible.

Chemical, biological and chromatographic methods are available to distinguish between these amines, but these methods are generally not sensitive enough for application to the low concentrations occurring in blood. Thus the measurable limits which can be attained in the set of five pharmacological tests devised by Gaddum, Peart & Vogt (1949) are given as 10 μg./l. for adrenaline and 100 μg./l. for noradrenaline.

Nevertheless, at our request, Dr T. B. B. Crawford of the Department of Pharmacology, University of Edinburgh, applied the rat-uterus method to a plasma eluate which we had sent. Its apparent adrenaline content had previously been estimated by our method and amounted to 3·0 μg./l. In the rat-uterus test adrenaline causes a depression of the contraction elicited by carbachol. The plasma eluate had a small effect in the wrong direction, since the response to carbachol was slightly increased. The addition of adrenaline to the eluate in a concentration of 2·5 μg./l. was more than enough to antagonize the slight stimulant action. A reagent blank did not affect the definite response of the rat uterus to a solution containing 2·5 μg. of adrenaline/l.

In our opinion, this result does not yet definitely exclude the identity of the plasma substance with adrenaline. As pointed out by Gaddum *et al.* (1949), blood contains interfering substances whose effect on the rat uterus is antagonistic to that of adrenaline. The eluate tested was not free of this interference and it is conceivable that the amount of preformed adrenaline was just adequate to counterbalance most of the antagonistic effect. It is, however, far more probable, especially in view of the results of von Euler & Schmiterlöw (1947), that the bulk of the plasma substance is identical with noradrenaline rather than adrenaline. Work is in progress to decide this problem by paper-chromatographic methods.

For many questions of clinical interest relative figures for the combined level of sympathomimetic amines in blood would be of value, provided there are no drastic changes in the proportion of the components. Such figures can be obtained in the clinical laboratory by the method described. Pending a final identification of the reacting material the results have been expressed in terms of adrenaline. To convert them to noradrenaline concentrations the figures have to be multiplied by 5.

The concentration of adrenaline-like substances in human blood

Fifty-four samples of blood were collected from the staff and patients of Runwell Hospital under ordinary conditions. The mean amine concentration corresponded to $3{\cdot}39 \pm 0{\cdot}043$ μg. adrenaline/l. plasma. Variations from the mean were comparatively slight, the range being from 2·9 to 4·3 μg./l.

The values for the normal adrenaline content of blood reported by previous authors are notoriously divergent (for reviews of the literature see Lehmann & Michaelis, 1942; Jörgensen, 1945; Blaschko, 1950). Most of them are much higher than those found by our method, a clear indication that the methods used were lacking in specificity. There are, however, some results, such as those of Bloor & Bullen (1941) and especially those recently obtained by Anrep, Barsoum & Gabrawy (1950), which broadly agree with ours, or such as those of Lund (1949*b*) which, at least, do not contradict them. Expressed as L-noradrenaline our results are in remarkably good agreement with the value of 10–20 μg./l. found by von Euler & Schmiterlöw (1947).

Some authors (Stewart & Rogoff, 1917; Trendelenburg, 1923; Guyton & Gillespie, 1951) tried to calculate the circulating concentration of adrenaline from a consideration of the rates of secretion and elimination or the amounts required to maintain blood pressure after adrenalectomy. They all arrived at figures in the neighbourhood of 1 μg./l. It seems that, in order of magnitude, these estimates which neglected extra-adrenal sources of sympathomimetic amines are confirmed by our observations.

The extremely high values obtained by Lehmann & Michaelis (1942, 1949) and by Annersten, Grönwall & Köiw (1950) in non-deproteinized plasma are attributed by these authors to a protein-bound, biologically inactive form of adrenaline. For this there is at present no experimental support. As pointed out by Lund (1949*b*), it is unlikely that the adrenaline-protein complex would remain undissociated when plasma is passed through an alumina column. There is certainly no retention of adrenaline when it is added to plasma.

Changes of plasma adrenaline concentration under various conditions

Electrical stimulation of the brain by external electrodes may increase the apparent adrenaline level in plasma by as much as 100%, whether convulsions occur or not. In general anaesthesia, on the other hand, the adrenaline content may fall to about 40% of the initial value.

Contrary to a widely held opinion a highly significant fall of the adrenaline level in blood is invariably observed during insulin hypoglycaemia. This phenomenon is fully developed within 30 min. after the intramuscular injection of insulin, long before consciousness becomes clouded. The effect is not due to an exhaustion of the adrenergic system, since an intravenous injection of glutamic acid, or the conduction of electric current through the brain, during hypoglycaemic coma, causes a pronounced rise of blood adrenaline.

These effects will be fully reported in a later publication.

SUMMARY

1. A fluorimetric method for the estimation of adrenaline-like substances in blood is described. It consists of the following steps: (*a*) filtration of plasma-buffer mixture (pH 8·4) through a column of acid-washed alumina and elution of the adsorbed amines by dilute acetic acid; (*b*) heating of the eluate at 50° with a mixture of ethylenediamine and ethylenediamine dihydrochloride; (*c*) extraction of a stable fluorescent condensation product with *iso*butanol; (*d*) measurement of fluorescence. The method has the advantage, compared with previous methods, that adrenochrome, a labile oxidation product of adrenaline formed as an intermediary, is trapped in the nascent state and quantitatively converted into a stable condensation product.

2. A series of recovery experiments showed that added adrenaline was quantitatively recovered with a standard deviation of about 5%. Concentrations $\geqq 1$ μg./l. may be determined by the method.

3. A study of the specificity of the method and of the action of amine oxidase led to the conclusion that the reactive material in blood consists entirely of amines derived from catechol. Whether this is adrenaline, noradrenaline or a mixture of both, remains to be further investigated.

4. Expressed in terms of adrenaline the mean concentration observed in human venous blood under normal conditions is about 3 μg./l. Since the fluorescence formed from noradrenaline is one-fifth of that produced by adrenaline, figures of adrenaline concentrations have to be multiplied by 5 to convert them to noradrenaline concentrations.

5. Some further results obtained with the method are briefly reported.

We are greatly indebted to Prof. J. H. Gaddum, F.R.S., and to Dr T. B. B. Crawford for their co-operation in carrying out pharmacological tests on a plasma eluate, and to Mr N. W. Ellis for help and advice concerning the adaptation of the Unicam spectrophotometer to fluorimetry. We also wish to acknowledge a gift of L-noradrenaline by Messrs Bayer Products Ltd. and of epinine and isoprenaline by the Wellcome Foundation Ltd.

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The Corneal Mucopolysaccharide

By A. M. WOODIN
Ophthalmological Research Unit (Medical Research Council)
Institute of Ophthalmology, London, W.C. 1

(*Received* 18 *August* 1951)

The acid mucopolysaccharide of cornea is present in the ground substance between the collagen fibres of the stroma. Although the presence of such a substance has been known since the work of Morner (1894) its chemical nature has been the subject of much discussion and the relation between the mucopolysaccharide and the collagen fibres has not been considered. Such information is of value for many reasons, amongst which can be mentioned the control of the permeability of the stroma, and the possible dependence of the transparency of the tissue on the relation between the mucopolysaccharide and the collagen fibres.

Meyer & Chaffee (1940), who summarized the earlier work on the corneal mucopolysaccharide, obtained a protein-free preparation from an extract of the water-insoluble residues of cornea. This preparation gave positive reactions for a uronic acid, for acetyl groups, and for ester-S; glucosamine hydrochloride was isolated from a hydrolysate. These substances were present in equimolecular amounts. Moreover, they found that the corneal mucopolysaccharide was a substrate for enzyme preparations having hyaluronidase activity, and they therefore suggested that it was a natural sulphuric acid ester of hyaluronic acid. The optical rotation was in keeping with this hypothesis, and they called the corneal mucopolysaccharide hyaluronosulphate.

Wislocki, Bunting & Dempsey (1947) found that the intensity of metachromasia in sections of cornea stained with toluidine blue was not reduced by treatment of the sections with hyaluronidase, but drew no conclusions from the observation. In a further paper (Dempsey, Bunting, Singer & Wislocki, 1947), they described the basophilia-pH curve of various tissues containing mucopolysaccharides. That of the cornea resembled those for tissues containing hyaluronic acid, in having the basophilia suppressed at pH 4. As an interpretation they suggested that the ester-S groups were not ionized in the intact tissue, or that they were absent from the mucopolysaccharide.

Werner & Odin (1949) analysed by paper chromatography a mucin from the aqueous extract, and an acid mucopolysaccharide obtained from the water-insoluble residues of cornea. Both preparations contained glucosamine, glucuronic acid, galactose, mannose and ester-S. Glucose was present in the digest from the aqueous residues but not in the mucin. They suggested that the cornea contains both a neutral and an acid mucopolysaccharide. It is difficult to evaluate their results as it is apparent that neither of their preparations was homogeneous and, although they suggested a progressive liberation of monosaccharide residues on acid hydrolysis, they did not report the position on their chromatograms of products of partial hydrolysis.

Evidence for the inactivity of hyaluronidase in the cornea was offered by Woodin (1950*a*), who found that enzyme preparations from several sources were without action as spreading factors, even under artificial conditions chosen to facilitate diffusion.

In this investigation, the stages in the extraction and purification of the mucopolysaccharide have been followed by a chemical and electrophoretic analysis in an attempt to determine the way in which the mucopolysaccharide is combined in the intact tissue. The action of hyaluronidase on the mucoids and mucopolysaccharide has been investigated, and a preliminary analysis of the mucopolysaccharide by paper chromatography has been attempted. Evidence so obtained shows that the corneal mucopolysaccharide cannot be described as a sulphuric acid ester of hyaluronic acid. It has been found further that the mucopolysaccharide is capable of forming complexes with proteins present in the tissues and it appears that, *in situ*, the mucopolysaccharide is in combination with proteins of the collagen group.

METHODS

Analytical methods

The analysis figures given in this paper refer to the dry, ash-free, material. The ash content of solids was determined after incineration with H_2SO_4. When solutions were analysed they were dialysed against 0·001 N-HCl and subsequently against distilled water. The dry weight was then determined by drying at 100° and it was assumed to be ash-free. In some cases this was confirmed by ashing.

Total nitrogen. Kjeldahl digestion for 8 hr. with a selenium catalyst was followed by distillation in the Markham still. The distillate was collected in 0·01 N-$Ba(OH)_2$ and titrated against 0·01 N-HCl with a mixed methylene blue-methyl red indicator.

Ester sulphate was determined by the gravimetric procedure of Lugg (1938) after hydrolysis in 5·8 N-HCl at 100° for 20 hr.; about 10 mg. of $BaSO_4$ were weighed and duplicates differed by 10%.

Hexosamine. This was determined by the modified Elson-Morgan reaction described by Pirie (1949). In the condensation reaction with acetyl-acetone the necks of the tubes were cooled with running water. Analytical figures given here refer to all the material which reacts with the Elson-Morgan reagents. In the early stages of this work, reported by Woodin (1950*b*), glucosamine standards were incorporated after the hydrolysis, but Ogston & Stanier (1950) have shown that glucosamine is destroyed when it is heated in acid solution and this result has been confirmed here. All results reported in this paper were obtained from standards which had been put through the hydrolysis procedure given to the corneal material.

pH 4–5 with $Ba(OH)_2$, and evaporated to dryness. About 300 μg. of the neutralized hydrolysate were added to the chromatogram. In some cases the neutralized hydrolysate was treated with ion-exchange resins before being added to the papers. Zeo-Karb 215 and De-acidite E were ground to a 40-mesh powder and 20 ml. wet vol. of each put into columns. The resins were washed and then regenerated with HCl and NH_4OH, respectively. The neutralized hydrolysate was diluted and 3 ml., containing 6 mg. of solid, pressed into the column. The non-absorbed sugars were washed out with 20 ml. water. A standard solution of galactose, glucosamine, and glucurone was put through the columns as a control.

When the amino-acid composition of the mucoids was required, they were hydrolysed in 6 N-HCl for 12 hr. and the acid removed *in vacuo*. Two-dimensional chromatograms were run in phenol-NH_3-KCN and in collidine. They were developed with ninhydrin.

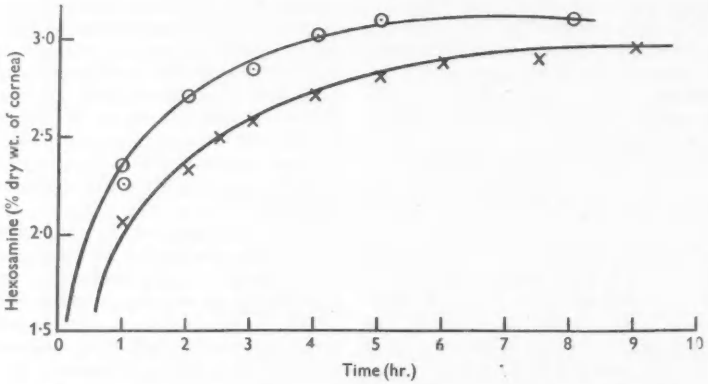

Fig. 1. Liberation of hexosamine from cornea on acid hydrolysis. ⊙, 5·8 N-HCl; ×, 3·48 N-HCl.

Fig. 1 shows the rate at which hexosamine is liberated from the cornea on acid hydrolysis. As a routine method the corneal material was hydrolysed in 5·8 N-HCl for 6 hr. at 100°.

Electrophoresis

This was done in the Tiselius apparatus at +2° with the optical system of Philpot (1938). The conductivity of the buffer solutions was measured at 0°. The pH was measured at 0° by a glass electrode calibrated against 0·05 M-phthalate buffer, pH 4·0. To reduce boundary anomalies and viscosity effects, solutions containing a high concentration of salt and a low concentration of colloid were used. The corneal material was dialysed against a buffer containing NaCl (*I* NaCl 0·18, *I* buffer 0·02) and then the solution diluted to give a refractive increment of $0{\cdot}75 \times 10^{-3}$.

Chromatography on filter paper

The techniques and purification of solvents were those described by Partridge (1948*a*). The chromatograms were developed with aniline hydrogen phthalate (Partridge, 1949), the Elson-Morgan reagents (Partridge, 1948*a*) and with the $AgNO_3$-KOH reagent (Trevelyan, Proctor & Harris, 1950). The mucopolysaccharide was hydrolysed in N-H_2SO_4 for 6 hr. or in 5 N-H_2SO_4 for 5 hr. The hydrolysates were brought to

Ionophoresis on filter paper

The apparatus described by Durrum (1950) was constructed and a 'power pack' used as the source of the current. Samples of the hydrolysate and of the standard sugars were put in line along the centre of a sheet of filter paper and the sheet draped over a glass rod with the line of spots as the apex. The apex was 25 cm. above the level of the electrolyte. A potential of 400–600 V. was then applied across the paper. After drying, the papers were developed with the aniline hydrogen phthalate, or the $AgNO_3$-KOH, reagents. No interference with the developers was found with 0·01 N-NH_3, 0·01 M-KH_2PO_4, 0·01 N-Na_2HPO_4 or 5 N-acetic acid, as electrolyte. In some cases the hydrolysate was first run as a partition chromatogram and then, after removing the solvent and adding the electrolyte, a potential was applied at right angles to the direction in which the chromatogram had been irrigated.

Hyaluronidase preparations

The enzyme preparations have been described previously by Woodin (1950*a*). The activity of the corneal mucopolysaccharide as substrate for hyaluronidase was assessed by measuring the reducing power of the solutions (Hagedorn & Jensen, 1923) after incubation with the enzyme. The solu-

tions of the corneal material were dialysed against a phosphate buffer containing NaCl (0·11 M-NaCl, 0·02 M-phosphate) before incubation. The pH of the buffer was 4·6, 5·5, 7·0, respectively, when testis, *Cl. welchii*, and streptococcal hyaluronidase, was used. The concentration of the corneal mucoids and mucopolysaccharide was deduced from the hexosamine content of the dialysed solutions.

Test of enzyme digestion

The dialysed substrate solution (2 ml.) was put in tubes and 0·1 ml. of the enzyme added. The mixed solution (0·5 ml.) was immediately withdrawn and added to the Hagedorn-Jensen reagent. The rest of the solution was incubated at 37° (see Table 5) and then 0·5 ml. withdrawn and added to the Hagedorn-Jensen reagent.

A crude preparation of hyaluronic acid from ox vitreous filtrate was digested in parallel. The filtered vitreous was precipitated with 3 vol. of acetone containing 3% acetic acid, and the precipitate collected, suspended in water, and dialysed. The insoluble material was rejected and the supernatant precipitated again with acid acetone. The precipitate was then dried. After dialysis against the appropriate buffer, the concentration of the hyaluronic acid was determined from the hexosamine liberated after hydrolysis in 5·8 N-HCl for 6 hr. at 100°.

Viscosity measurements

These were made at 25° in an Ostwald viscosimeter having a flow time of 20 sec. for 3 ml. water. The corneal mucoids were dialysed against a phosphate-NaCl buffer at pH 7 (*I* NaCl 0·18, *I* phosphate 0·02). The concentration of the solutions was determined from their nitrogen content. For each substance investigated, a curve was constructed relating the concentration to the log. of the relative viscosity. This relationship was linear and in the section describing the results the viscosity of the mucoids will be given as log $\eta_{rel.}$ for a 0·5% solution.

Preparation of material

The corneas were cut from ox eyes 2–3 hr. after the death of the animal, rinsed in distilled water, and frozen into blocks. They were then cut to 20 μ. sections on the freezing microtome. The sections could then be dried by sublimation, or by acetone, or could be extracted directly.

Extraction of the cornea

In general a known weight of the sections (of which the dry weight and ester-S content was known) was suspended in the solvent using about 25 ml. for each gram, dry weight, of the sections. The suspension was brought to pH 8, left for 3–4 days at 0°, and then centrifuged and the residues extracted again twice. Finally the residues were dialysed till free from Cl^- and their dry weight and ester-S content determined.

Sulphatase activity

If the extraction experiments were to be of value, it was important to know whether the corneal sections had a sulphatase activity. 1 g. sections, dried by sublimation, was suspended in water, the pH of the suspension being 6·7, and then incubated at 37° in the presence of toluene for 24 hr. A further sample was heated to 100°, 25 ml. of boiling water added and the suspension kept at 100° for 15 min., then cooled and incubated at 37° for 24 hr. At the end of the incubation period the tubes were filtered, the filtrates acidified and the mucin was centrifuged off. 0·5 M-$BaCl_2$ (1 ml.) was then added to 15 ml. of the supernatant. A visible precipitate of $BaSO_4$ was produced in each case, but neither weighed more than 200 μg.

RESULTS

Composition of the cornea. The sections of cornea, after washing in acetone, contained 0·25–0·26% ester-S and 3·00–3·25% hexosamine. The ester-S corresponds to a mucopolysaccharide content of 4·2%. On the basis of their final yield, Meyer & Chaffee (1940) gave the mucopolysaccharide content of the cornea as 1·8%. Only 54% of the hexosamine of the cornea is accounted for as a component of the mucopolysaccharide. Under the conditions of the test, the corneal sections had no sulphatase activity. The inorganic sulphate in the cornea represents less than 5% of the ester sulphate.

Extraction of the mucoids from cornea. Meyer & Smyth (1937) used 10% calcium chloride to extract acid mucopolysaccharides from connective tissues. Partridge (1948*b*) obtained good yields of chondroitin sulphate by extracting cartilage with 10% calcium chloride. Extraction with sodium chloride at pH 8 is the basis of the Bergman & Stein (1939) method of preparing collagen and was used by Pirie (1947) to free corneal collagen from the ground substance. These methods have been compared here. Initially the tissue was dehydrated with acetone to denature water-soluble proteins, but it has been found that drying the tissue reduces the efficiency of the extraction. A better yield was obtained from sections which had not been dried. The most efficient extraction was from sections which had swollen in water to give a wet weight/dry weight ratio of 32.

Table 1. *Extraction of cornea at* pH 8

(Where sections were extracted successively by different solvents the amount of mucoid dissolved, given in column 3, is the percentage of that initially present in the intact tissue. The amount in the residues is that remaining after extraction with all solvents.)

Nature of sections	Solvent	Ester-S dissolved (%)	Ester-S in residues (%)
Dry	10% $CaCl_2$	45–50	0·2
Wet	10% $CaCl_2$	60–65	0·1
Wet	(*a*) 10% NaCl	40	—
	(*b*) 10% $CaCl_2$	30	0·09
Wet	(*a*) Water	20	—
	(*b*) 10% NaCl	30	—
	(*c*) 10% $CaCl_2$	35	0·05
Swollen	10% $CaCl_2$	85	0·06

The results of the extraction experiments are given in Table 1. The same efficiency of extraction of

dry sections was found whether drying was by acetone at room temperature or at $-10°$, or by sublimation. Heating the sections at 45° for 30 min. (Partridge, 1948*b*) did not increase the yield of mucoid.

More mucoid could be dissolved from the residues after extraction at pH 8 by heating them at 37° and pH 11 for 30 min. Although a mucoid rich in ester-S could be recovered by fractionation of this extract, the residues from the extraction at pH 8 and at pH 11 contained the same amount of ester-S. It appears that protein and mucopolysaccharide are dissolved to the same extent at pH 11.

ethanol at $-10°$. These substances will be called the 'insoluble mucoids', and the 'soluble mucoids', respectively. The insoluble mucoids were washed with water and the suspension frozen and dried by sublimation. The soluble mucoids were dissolved in water and dried by sublimation.

We shall be concerned with the extracts made from acetone-dried sections, from water-swollen sections, and from the residues remaining after extraction of the sections with water. Only the extracts made in calcium chloride solution have been studied to any extent. The soluble mucoids and the insoluble mucoids will be described separately. The soluble

Table 2. *Composition of corneal extracts*

	N (%)	Ester-S (%)	Hexosamine (%)
(1) Extract made in distilled water	13·1	0·53	7·3
(2) Direct precipitation with 3 vol. ethanol and 0·5% sodium acetate of extract made in 10% $CaCl_2$:			
From dry sections pH 8	13·4	0·70	8·8
From fresh sections pH 8	12·3	0·70	9·0
From fresh sections pH 8 with heat treatment	14·6	—	7·5
From fresh sections pH 11 (after pH 8 extraction)	14·8	0·80	5·0
(3) Water-soluble mucoids prepared by 1 vol. ethanol precipitation of dialysed $CaCl_2$ extract, pH 8:			
From dry sections	11·5	1·1	11·4
From swollen sections	11·4	1·3	11·7
From water-extracted sections	11·2	2·1	12·7
(4) Water-soluble mucoids from dialysed $CaCl_2$ extract, not fractionated with ethanol:			
From dry sections	12·2	0·73	8·2

Table 3. *Electrophoresis of soluble mucoids*

(The refractive increment of the solution was about $0·75 \times 10^{-3}$ in a buffer of composition *I* NaCl 0·18, *I* buffer 0·02. A current of 0·01–0·02 amp. was passed for 6–8 hr.)

Origin	pH	No. of components	Contribution of fastest to total refraction (%)	Hexosamine/nitrogen ratio of fastest component
Dry sections	9·05	2	60	1·3
	6·80	2	62	1·3
Swollen sections	6·8	4	50	1·4
	4·7	3	50	—
Water-extracted sections	6·8	2	62	2·5

Fractionation of the extracts. In the earliest stages of this work the extracts were precipitated directly with 3 vol. ethanol and sufficient sodium acetate to give a final concentration of 0·5%. The solid obtained had a high ash content and was only partly soluble in water and dilute salt solutions; moreover, it gave unsatisfactory results when fractionated by the methods described below. The composition of the products of direct precipitation is given in Table 2.

Subsequently, the extracts made in salt solutions were dialysed against running tap water and then against distilled water. A precipitate appeared during the dialysis and material remained in solution which could be recovered by the addition of 1 vol. of

mucoids account for 85% of the total amount of ester sulphur extracted from the swollen sections, and for 80% of the total extracted from the dry sections. Fig. 2 summarizes the fractionation procedures described below.

The soluble mucoids

The composition of the soluble mucoids is given in Table 2. Solutions of the soluble mucoids were examined in the Tiselius apparatus since it was hoped to prepare a protein-free preparation of the mucopolysaccharide by the electrophoretic method. However, as Table 3 indicates, the fast components of the mucoids always contain considerable amounts

of protein. The mobility of the slowest component was very low and these results are not evidence for its homogeneity.

represent part of the water-soluble proteins of the cornea. They are absent from the extract from dry sections, perhaps because of denaturation by the

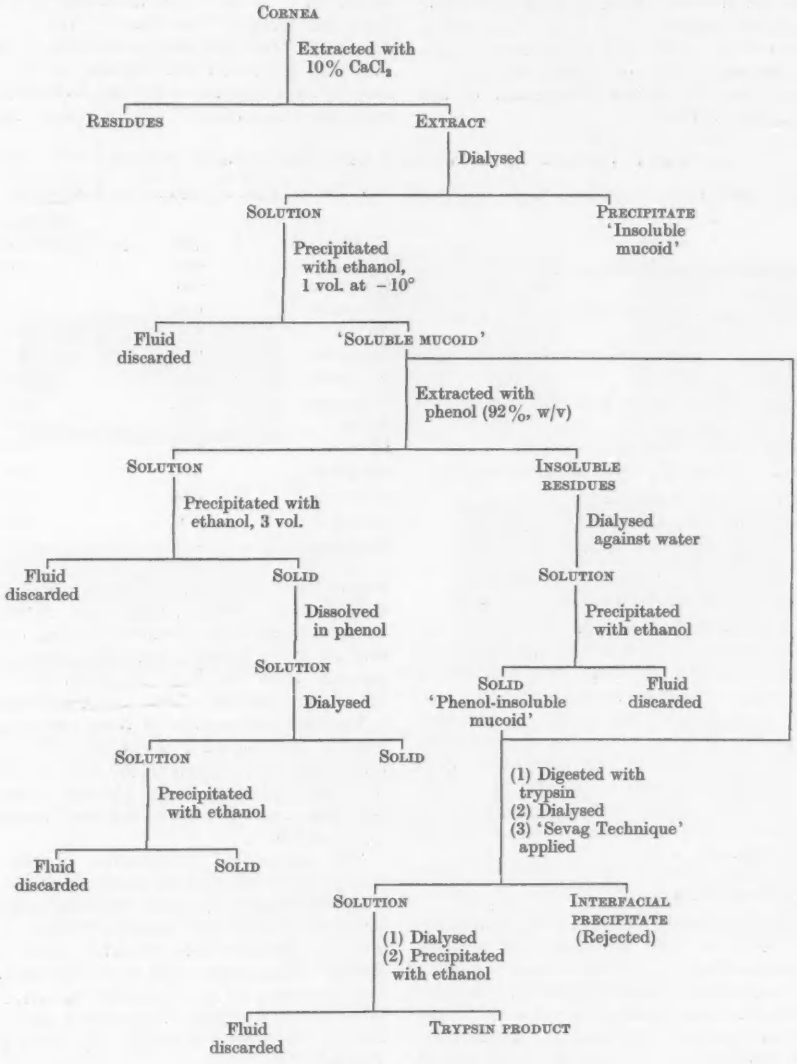

Fig. 2. Diagram of fractionation procedure.

At pH 4·7 the fast component of the soluble mucoids from the swollen sections contained 11·0% nitrogen and the other components contained 12·0% nitrogen. The two components of intermediate mobility present in this extract probably

acetone, and from the soluble mucoids from the aqueous residues because of the preliminary water extraction. Preliminary experiments on the water-soluble fraction of the corneal proteins support this suggestion. The aqueous extract had four com-

ponents at pH 6·8, the fastest being responsible for 20 % of the total refraction and having a mobility of the same order as that of the soluble mucoids from dry and swollen sections. When the aqueous extract was precipitated with 3 vol. of ethanol and 0·5 % sodium acetate at −10°, the precipitate did not dissolve when suspended in water and dialysed.

The mobilities of the fast components of the mucoids is given in Table 4.

The phenol-insoluble fraction from dry sections separated into two components on electrophoresis at pH's 6·8 and 3·97. The slow component represented 25 % of the total refraction, of which an unknown amount was due to the 'boundary anomalies'. The slow component was not identical with the phenol-soluble fraction of the soluble mucoids since it persisted through successive phenol extractions and ethanol precipitations. A sample was isolated and found to contain about 5–10 % hexosamine. The phenol-insoluble material from the swollen sections contained a small amount of this slow component, but the phenol-insoluble material from the water-extracted sections contained less than 2 % of a slow-moving component.

Table 4. *Electrophoretic mobilities of fast components of the mucoids*

(The electrolyte had the composition *I* NaCl 0·18, *I* buffer 0·02. The mobilities are means from both limbs.)

Origin	Buffer	pH	Mobility $\mu \times 10^5$ (cm.^{2} sec.$^{-1}$ V.$^{-1}$)
Soluble mucoids from acetone-dried sections	Glycine	9·05	−7·6
	Phosphate	6·80	−7·4
Soluble mucoids from swollen sections	Phosphate	6·80	−7·8
	Acetate	4·7	−7·2
Soluble mucoids from water-extracted residues	Phosphate	6·82	−9·12
Aqueous extract	Phosphate	6·80	−8·05
Phenol-insoluble mucoid from acetone-dried sections	Phosphate	6·90	−9·0
	Acetate	3·97	−8·6
Phenol-insoluble mucoid from swollen sections	Phosphate	6·25	−9·2
Phenol-insoluble mucoid from water-extracted residues	Phosphate	6·82	−9·4
Trypsin product from dry sections	Phosphate	6·80	−9·25
Trypsin product from swollen sections	Phosphate	6·64	−9·4
Trypsin product from aqueous residues	Phosphate	6·62	−9·4

Extraction of the soluble mucoids with phenol

That polysaccharides can be separated from proteins by extraction with phenol was first shown by Morgan & Partridge (1941), for mucoids of bacterial origin. Phenol has subsequently been used to reduce the protein content of mucoids from connective tissues (Rogers, 1945; Partridge, 1948*b*). When the soluble mucoids from all three types of corneal sections were extracted with 92 % (w/v) phenol at room temperature, a separation was achieved into a fraction soluble in phenol and one insoluble in phenol.

Phenol-insoluble fraction

The insoluble fraction was suspended in water and dialysed, when it gave an opalescent solution. A solid was precipitated by the addition of 2 vol. of ethanol, redissolved in water and dried by sublimation. The solid was extracted again with phenol, dialysed, precipitated with ethanol, dialysed, and dried by sublimation. So prepared, the phenol-insoluble material had the composition:

From dry sections: 8·0 % N, 18·4 % hexosamine, 3·6 % ester-S.

From swollen sections: 8·3 % N, 19·0 % hexosamine.

From water-extracted sections: 8·7 % N, 21·0 % hexosamine.

The fast components of these phenol-insoluble mucoids had similar mobilities (see Table 4), and a Hexosamine/Nitrogen (H/N) ratio of 2·5–3·0. A 0·5 % solution of the fast component of the phenol-insoluble material from the dry sections had $\log \eta_{rel.} = 0{\cdot}28$.

The amino-acid composition of the phenol-insoluble mucoid from the water-extracted residues was determined by paper chromatography. The presence of glycine, alanine, proline, hydroxyproline, glutamic and aspartic acids, arginine, lysine, valine, serine and threonine, and an area corresponding to phenylanaline and the leucines could be demonstrated. These amino-acids are those shown to be present in collagen by Bowes & Kenten (1949).

The results described above refer to mucoids obtained by extraction with calcium chloride solutions. When the water-insoluble residues of the cornea were extracted with 10 % sodium chloride, pH 8, water-soluble mucoids were obtained with 1·9 % ester-S. This, at pH 6·8, had a fast component in the Tiselius apparatus which was responsible for

55 % of the total refraction. When the dry mucoids were extracted with 92 % phenol, the nitrogen content of the insoluble fraction could not be reduced below 8·0 %. It is hence unlikely that the association between the polysaccharide and the protein in the corneal extracts is due to a specific action of the Ca^{++} ion.

Phenol-soluble fractions

The first phenol extract of the soluble mucoids gave a precipitate on addition of 3 vol. of ethanol, but only a slight turbidity was obtained from the second phenol extract. The solid precipitated from the phenol solution was dried and dissolved again in phenol. Dialysis of this solution against water gave a precipitate and a solution from which material was precipitated by the addition of 2 vol. of ethanol.

The solid produced by the addition of ethanol to the phenol extract of the soluble mucoids from the dry sections contained 0·25 % ester-S, 2·6 % hexosamine, 13 % nitrogen. The solid precipitated during the dialysis of the phenol extract of the soluble mucoid from the water-extracted sections contained 14·8 % nitrogen. The water-soluble, phenol-soluble, fraction of the same origin had a H/N ratio of 1·4.

The hydrolysate of the phenol-soluble, water-insoluble fraction derived from the water-insoluble residues of cornea had an amino-acid composition similar to that of the phenol-insoluble fraction and it would appear that both are derived from corneal collagen.

Treatment of the phenol-insoluble material with chloroform and amyl alcohol

An attempt was made to reduce the protein content of the phenol-insoluble mucoid by the 'Sevag' technique. 0·3 % solutions of the phenol-insoluble material from dry sections were made in 0·25 M-NaCl, pH 4; 0·25 M-NaCl, pH 8; and 10 % $CaCl_2$, pH 8, and each was shaken with a mixture of 4 vol. of chloroform and 1 vol. amyl alcohol. The interfacial precipitate from the successive shakings was collected, and washed with distilled water by centrifugation, till the supernatant was free from Cl^-. The precipitate contained 7·5–8·0 % N (not corrected for ash content). It appeared that the phenol-insoluble mucoid was precipitated unchanged.

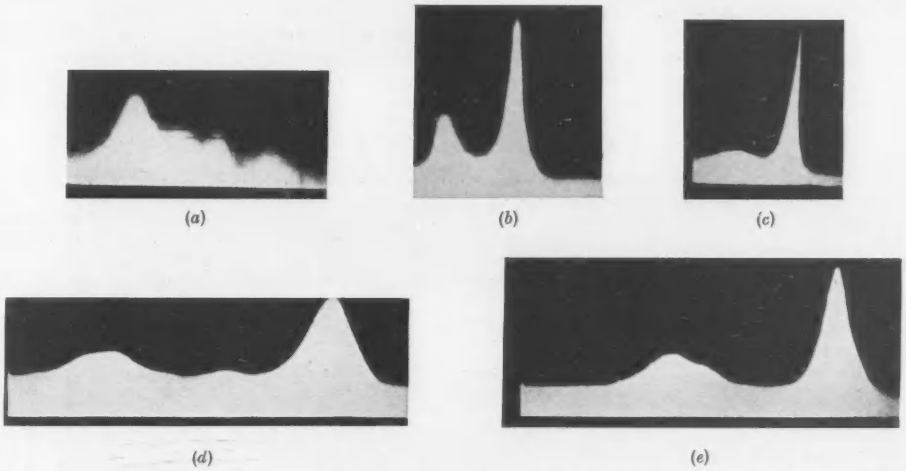

Fig. 3. Electrophoretic diagrams of corneal extracts. In all cases the buffer had the composition *I* NaCl 0·18, *I* buffer 0·02. The photographs have been taken with the slit at a high angle and are of the descending limbs with migration from left to right. (*a*) Aqueous extract, pH 6·8, 3 hr. from start. (*b*) Soluble mucoids from dry sections, pH 6·8, 2 hr. from start. (*c*) Phenol-insoluble mucoids from swollen sections, pH 6·8, 2·5 hr. from start. (*d*) Soluble mucoids from swollen sections, pH 6·8, 4 hr. from start. (*e*) Soluble mucoids from swollen sections, pH 4·7, 3 hr. from start.

Treatment of the mucoids with trypsin

Solutions of the phenol-insoluble material, and of the soluble mucoids, were adjusted to pH 8 and incubated at 37° with crystalline trypsin until the pH remained constant. About 10 mg. of trypsin were added for every 250 mg. of hexosamine present in the mucoids. A precipitate which appeared in the digest during the incubation was centrifuged off, and the supernatant dialysed. The N content of the soluble material could be reduced to 4–5 % by this process. The dialysed digest was diluted to give an 0·2 % solution in 10 % $CaCl_2$, pH 8, and shaken with chloroform and amyl alcohol. When no further interfacial precipitate appeared, the process was repeated at pH 4 and the interfacial precipitate

removed. The solution was finally dialysed against 0·001 N-HCl, precipitated from the acid solution by 2 vol. ethanol, redissolved in water, dialysed, and dried by sublimation. After dialysis against 0·001 N-HCl and precipitation from the acid solution with ethanol, 10 mg. had no weighable ash so that analytical figures refer to the free acid and not to a salt.

The nitrogen content of the 'trypsin products' varied between 2·9% and 4·0%, and an electrophoretic examination revealed that they were not homogeneous. Preparations from all three types of corneal section had a fast-moving component and a material of very low mobility. In the trypsin product from the water-extracted sections, this slow component was responsible for 25% of the refraction, and in the other products, rather more. The fast component contained 3·3% nitrogen, 35% hexosamine, and 6·2% ester-S. The fast component of the trypsin product from the dry sections had $\log \eta_{rel.} = 0{\cdot}14$ for a 0·5% solution.

Analysis by chromatography on filter paper

Analysis of mucopolysaccharides which contain uronic acids and hexosamines is complicated by the fact that the hydrolysis can proceed in stages with destruction of the more labile residues before all the glycosidic linkages are broken. Both degradation products and products of partial hydrolysis can be expected in the hydrolysate.

The analyses reported below were made on the fast component of the trypsin product, isolated after electrophoresis at pH 6·8. The same results have been obtained whatever the origin of the trypsin product. The hydrolysis conditions used have been N-sulphuric acid at 100° for 6 hr. and 5N-sulphuric acid at 100° for 6 hr. The polysaccharide yielded 78% reducing substances (as glucose) after the hydrolysis with 5N acid; with the N acid hydrolysate the reducing power was 38%. It was found that all the ester-S was removed by hydrolysis in N-hydrochloric acid for 6 hr. at 100°, and it is presumed that sulphuric acid has the same effect.

Table 5. *Hydrolytic action of hyaluronidase*

(All the corneal substrates were derived from acetone-dried sections. The liberation of less than 2·5% of the total reducing sugar is not significant and has been neglected in column 5.)

Substrate	Enzyme	Incubation time	Reducing sugar available (mg.)	Total reducing sugar liberated (%)
Hyaluronic acid	Streptococcal	1 hr.	1·18	24
	Cl. welchii	1 hr.	1·18	8
Phenol-insoluble mucoids	Streptococcal	1 hr.	1·48	0
	Cl. welchii	1 hr.	1·48	0
Hyaluronic acid	Streptococcal	49 min.	2·61	19
	Testis	49 min.	1·69	9
Fast component of phenol-insoluble mucoids	Streptococcal	6 hr.	2·05	0
	Testis	6 hr.	2·05	0
Trypsin product	Streptococcal	4 hr.	2·06	0
	Testis	4 hr.	2·06	0

Action of hyaluronidase on the mucoids and mucopolysaccharide

Table 5 gives the results of incubating the corneal extracts with hyaluronidase. The depolymerase activities of the enzyme preparations were such that 0·1 ml. of all enzymes tested disaggregated the hyaluronic acid in 2 ml. of the filtrate from ox vitreous body in less than 60 sec. That they possessed a hydrolytic activity is shown by the reducing sugars liberated from the hyaluronic acid preparation. The reducing sugar available in the hyaluronic acid preparation was calculated from the apparent hexosamine content; it is probable that hyaluronic acid is not the only substance containing hexosamine in this preparation (Pirie, 1949), and the figures given for the percentage of hydrolysis are probably too low. In calculating the percentage hydrolysis of both the hyaluronic acid and the corneal material, the assumption was made that reducing sugar equivalent to 2 equiv. of glucose was present for every equivalent of hexosamine.

Chromatogram irrigated with collidine

After addition of the hydrolysate and the standard sugars to the paper, 5N-ammonia was added to each spot and allowed to dry off at room temperature. The paper was then irrigated for 48 hr. On development, the presence of galactose, glucosamine, and galactosamine in, and the absence of glucurone and glucuronic acid from, both hydrolysates could be demonstrated unambiguously. There was also present in the N-sulphuric acid hydrolysate, a substance which extended from the base line to a position 0·2 the distance moved by galactose, and which reacted with all three spraying reagents. It was absent from the hydrolysate treated with Zeo-Karb and reduced in intensity in that treated with De-Acidite. The 5N-sulphuric acid hydrolysate also contained some reducing material of low mobility

which gave a diffuse trail from the base-line to a position 0·3 the distance moved by galactose.

Chromatogram irrigated with phenol

Galactose and a hexosamine were present in the N-sulphuric acid hydrolysate, and also material of low mobility which after 36 hr. irrigation separated into two spots and reacted with all three spraying reagents. The slower spot moved 0·15 the distance moved by galactose and was absorbed by the Zeo-Karb but not by De-Acidite. The faster moved 0·35 the distance moved by galactose and was absorbed by both resins. No amino-acids could be detected in 0·5 mg. of polysaccharide hydrolysed in 5N-sulphuric acid.

a potential across the paper with 0·01 N-ammonia as electrolyte. One of the slow-moving reducing substances moved to the anode.

No glucurone was present in the hydrolysate. Its absence is probably not due to destruction since a glucurone spot was found in a chromatogram from a mixture of galactose, glucurone and glucosamine, which had been put through the N-sulphuric acid-hydrolysis procedure.

Ionophoresis on filter paper

After the addition of the hydrolysate, or the standard sugars, to the papers 5N-ammonia was added to the spots and allowed to dry off at room temperature. This was a necessary procedure to

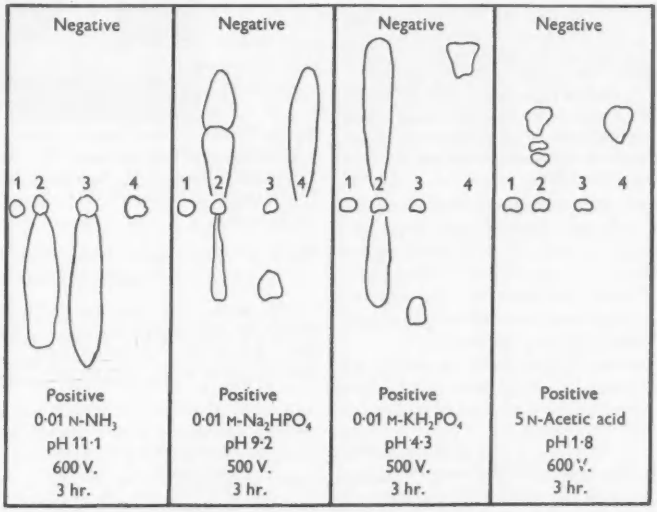

Fig. 4. Diagram of papers developed with aniline hydrogen phthalate after ionophoresis. 50 μg. of the standard sugars and 300 μg. of the hydrolysate were added to the spots. Positions are relative to galactose and not to the initial position. 1 = Galactose; 2 = N-H_2SO_4 hydrolysate; 3 = glucurone + NH_3; 4 = glucosamine hydrochloride.

Chromatogram irrigated with butanol-acetic acid

After 36 hr. irrigation, galactose and glucosamine were not separated. The N-acid hydrolysate showed in addition to these substances two reducing materials, the slower moving 0·35 as far as galactose, and the faster 0·59 as far as galactose. The slower was removed by both resins, the faster by the Zeo-Karb only. That the absorption by the De-Acidite was due to a negative charge on the molecule and not to an unspecific absorption was shown by first irrigating the chromatogram with butanol-acetic acid and then, after removal of the solvent, applying ensure the conversion of some of the glucurone to ammonium glucuronate.

The results are summarized in Fig. 4. Glucosamine is not charged at pH 11 and glucuronic acid could not be detected in the glucurone at pH 1·8. It is clear that there is a negatively charged sugar ion in the polysaccharide hydrolysate. Acetic acid was the only solvent in which the hydrolysate gave discrete spots on ionophoresis. In the other solvents the charged species in the hydrolysate gave pear-shaped trails. The separation of the positively charged material into three spots with acetic acid as solvent is of interest; such an effect would be

expected if the hydrolysate contained partial hydrolysis products, or degradation products, in which the hexosamine was combined with neutral or weakly acidic substances.

Fractionation of the insoluble mucoids

The composition of the insoluble mucoids obtained from dry sections by calcium chloride extraction was 15% nitrogen, 3·5% hexosamine, 0·6% ester-S. The insoluble mucoids from sodium chloride extracts had a similar composition.

At least 0·5% sodium chloride was necessary to keep the insoluble mucoid in solution. As the solutions were very viscous, even in the presence of this concentration of salt, they were not analysed electrophoretically. The insoluble mucoids cannot be digested by trypsin.

The insoluble mucoids from the dry sections were dissolved in 10% $CaCl_2$ and the solution precipitated with 3 vol. of ethanol and a final concentration of 0·5% sodium acetate. The precipitate contained 13·5% N, and 6·0% hexosamine. The dry solid was extracted with 92% phenol solution at room temperature. The insoluble fraction was suspended in water and dialysed, when it dissolved to give an opalescent solution, from which the addition of 2 vol. of ethanol precipitated a white solid.

The ethanol precipitate contained 9% nitrogen, 17% hexosamine, and contained ester-S. A hydrolysate made in N-sulphuric acid showed glucosamine and galactose but no glucuronic acid. Moreover, this hydrolysate contained the slow-moving reducing material present in the N-sulphuric acid hydrolysate from the trypsin product.

However, the chromatograms were not so clear as those from the trypsin product and it was not possible to demonstrate the presence of galactosamine conclusively.

Phenol-soluble fraction of the insoluble mucoids

The phenol extract of the insoluble mucoids was dialysed against water and the precipitate collected; the suspension in water was frozen and the water sublimed off; all the solid redissolved in phenol and was precipitated by 3 vol. of ethanol. This solid contained 16·8% nitrogen.

It appeared from some of its properties that the protein component of the insoluble mucoids was derived from a collagen. It was thought that a test of this origin of the protein would be its further degradation to gelatin. Accordingly, the solid precipitated, by dialysis, from the phenol extracts of the insoluble mucoid was suspended in distilled water (pH 5·5) and heated at 105° for 4 hr. All but a small fraction dissolved to give a clear solution, which set to a gel on cooling, when the concentration was greater than about 1·5%.

This protein was examined in the Tiselius apparatus. To enable comparison with the mobilities of gelatin derived from the collagen in cartilage, the buffer solutions were the same as those used by Partridge (1948*b*). As the mobilities were low, results of the highest accuracy would require a buffer of smaller ionic strength. The protein was not homogeneous (Fig. 5). The difference in the mobility of the components was small, but was most marked in acetate buffers in the range pH 3–5. Table 4 gives the mobilities of the fast component; on the acid side of the isoelectric point they differ slightly from those for gelatin from cartilage.

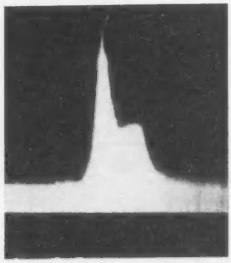

Fig. 5. Electrophoresis diagram of the gel-forming protein from the insoluble mucoids. The photograph is of the ascending limb, with migration from right to left. Acetate-NaCl buffer, pH 3·74. 7 hr. from start, current 0·01 amp.

Table 6. *Electrophoretic mobilities of the gel-forming protein from the insoluble mucoid*

(The buffer contained NaCl (I NaCl = 0·18, I buffer = 0·02). The mobilities are the means of the values from each limb. Where the boundaries split into two peaks the mobility refers to the fast component.)

Buffer	pH	Mobility ($\mu. \times 10^5$)
Phosphate	6·80	−1·0
Phosphate	6·80	−1·1
Phosphate	5·34	−0·85
Acetate	4·92	−0·52
Acetate	3·95	+1·28
Acetate	3·74	+1·44
Glycine	2·75	+2·95
Glycine	2·70	+2·65

Paper chromatography of a hydrolysate from the gel-forming proteins from cornea showed that the amino-acid composition was very similar to that of gelatin.

DISCUSSION

About 70% of the acid mucopolysaccharide has been extracted in an electrophoretically homogeneous form. The residues remaining after extraction of the swollen sections contain about 15% of the total ester-S and nothing is known about this fraction. The insoluble mucoids contain about 10–15% of the total ester-S and there is not sufficient evidence to permit identification of the carbohydrate components of the two mucoids. The

only evidence for the homogeneity of the mucopolysaccharide is the electrophoretic measurements. The chemical fractionation was designed to remove protein and the persistence of the mucopolysaccharide through this is not evidence for its homogeneity.

The identification of galactose as a constituent of the corneal mucopolysaccharide is of interest. Suzuki (1939) considered that galactose was present but, as he used an unspecific method of identification, Meyer & Chaffee (1940) considered that he had confused galactose and a uronic acid. I have not found a uronic acid as a constituent of the N-acid hydrolysate of the mucopolysaccharide. However, the lability of the uronic acids and the resistance of hexosaminosides to acid hydrolysis is well known, and it is possible that its absence is due to both destruction and insufficient hydrolysis. Moreover, one of the partial hydrolysis products has an acid group and that may be due to the presence of combined uronic acid. It is probable that the identification of the uronic acid stated to be present in the corneal mucopolysaccharide will be best achieved by protection of the labile groups of the molecule before hydrolysis.

It appears from the results of Kaye & Stacey (1951) that galactose is not a constituent of hyaluronic acid, and accordingly the suggestion of Meyer & Chaffee (1940) that the corneal mucopolysaccharide is a sulphate ester of hyaluronic acid is invalidated by the results described in the present paper.

The mucopolysaccharide is not hydrolysed by hyaluronidase preparations. The failure of the enzyme to have a spreading action in the cornea has already been reported (Woodin, 1950*a*), and it was suggested in that paper that an explanation of the failure to confirm the results of Meyer & Chaffee would be that the pneumococcal hyaluronidase used by these authors contained enzymes other than hyaluronidase. Pneumococcal infection produces a characteristic spreading ulcer in the cornea and presumably the spread is effected by enzymic processes.

The extraction experiments were designed primarily to determine the optimum conditions to bring the mucoid into solution in an undegraded form. Although a knowledge of the effect on the cornea of calcium chloride and sodium chloride in varying concentrations will be required before an explanation of these results can be offered, it is of interest that the effect of sodium chloride and calcium chloride in extracting the mucopolysaccharide from the cornea should parallel the effects of these substances in reducing the shrinkage temperature, and increasing the swelling pressure of the collagen in rat-tail tendon and in sheep skin respectively. Bowes & Kenten (1950) have suggested that the decrease in the cohesion brought about by calcium chloride is primarily due to a reduction in the potential energy of the inter-molecular links between the polypeptide chains. The results presented here suggest that the cohesive forces of the collagen fibres and the forces holding the mucopolysaccharide to the corneal collagen are very similar. It would be of great interest to know if the mucopolysaccharide of the cornea were contributing to the cohesion of the cornea collagen, since the orderly arrangement of the collagen fibres is probably a necessary condition of the transparency of the tissue.

The corneal mucopolysaccharide can form stable complexes with proteins. The fast component of the phenol-insoluble mucoids, which contains about 40 % protein, is homogeneous electrophoretically in the range pH 8–4 and is resistant to chemical fractionation by the 'Sevag' technique and by extraction with phenol. The fast component of the soluble mucoids from the water-extracted residues, and the phenol-insoluble material, have the same mobility and H/N ratio and they are probably identical. However, the fast component of the soluble mucoids from dry sections and from the swollen sections contains more protein than this complex; it is homogeneous in the range pH 9·05–4·7, and chemical combination can be presumed to occur between the protein and mucopolysaccharide components. The mobility of this fast component is smaller than that of the phenol-insoluble material, and it is at least possible that this soluble mucoid contains a complex formed by polar association between the phenol-insoluble material and some of the water-soluble corneal proteins.

It is of interest that the proteins with which the mucopolysaccharide is associated have an amino-acid composition qualitatively the same as that of gelatin. This fact, considered in conjunction with the results of the extraction experiments, suggests that the unit in the intact stroma is a mucopolysaccharide-collagen complex.

SUMMARY

1. The ester-S present in the cornea corresponds to a mucopolysaccharide content of 4·2 %. There is an excess of hexosamine over that required as a constituent of the mucopolysaccharide.

2. By extraction of the water-swollen sections of cornea with 10 % calcium chloride at pH 8, 85 % of the mucopolysaccharide can be dissolved. Extraction with calcium chloride is more efficient than extraction with sodium chloride and that is more efficient than extraction with water.

3. The mucopolysaccharide is extracted as a component of both water-soluble and water-insoluble mucoids.

4. The protein content of the water-soluble-mucoids can be reduced by extraction with phenol

to leave a phenol-insoluble mucoid. This contains a substance with the characteristics of a mucopolysaccharide-protein complex.

5. The water-soluble mucoids contain a complex which is formed by interaction between the phenol-insoluble mucoids and a water-soluble protein of the cornea.

6. The acid mucopolysaccharide can be prepared free from protein by incubation with trypsin. Galactose, glucosamine and galactosamine have been identified as constituents of the mucopolysaccharide, by chromatography and ionophoresis on filter paper.

7. The corneal mucopolysaccharide is not a substrate for hyaluronidase.

8. Gel-forming proteins have been isolated from the insoluble mucoid and found to contain the same amino-acids as gelatin, but to differ from it in their electrophoretic behaviour.

9. It is suggested that *in situ* the acid mucopolysaccharide is combined with proteins of the collagen group.

I wish to thank Dr A. S. McFarlane for allowing me to make the electrophoretic measurements in his laboratory and Mrs A. Dovey for much advice on the use of the instrument.

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The Synthesis of Cozymase from Nicotinic Acid and its Derivatives by *Lactobacillus arabinosus* 17–5

By D. E. HUGHES and D. H. WILLIAMSON
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield 10

(*Received* 7 *November* 1951)

As shown in previous papers from this laboratory (McIlwain, 1949; McIlwain, Stanley & Hughes, 1949), glycolysis by washed suspensions of *Lactobacillus arabinosus* 17-5, which have been grown on a nicotinic acid-deficient medium, is stimulated by the addition of nicotinic acid. The stimulation is due to the synthesis of cozymase and, since this organism does not rapidly decompose the coenzyme (McIlwain & Hughes, 1948), it seemed to be suitable material in which to study its synthesis. In the present studies, the effect of limiting amounts of nicotinic acid, nicotinamide and nicotinamide nucleotides on the stimulation of glycolysis and on the synthesis of cozymase has been investigated. Young deficient cells glycolysing in an optimal concentration of nicotinic acid rapidly synthesized cozymase until the cells became saturated with the coenzyme; synthesis of cozymase and metabolism of nicotinic acid then ceased. Glucose alone of the constituents of the growth medium was essential for the synthesis of cozymase by washed suspensions. The uptake of nicotinic acid ran parallel to the synthesis of coenzyme and no detectable amounts of any possible intermediate in the synthesis were detected in cells or the suspending fluid. Unexpectedly, nicotinamide was found to be rapidly deamidated by the

washed suspensions; deamidation was independent of the glucose and of the cozymase content of the cells.

EXPERIMENTAL

Growth conditions for obtaining cozymase-deficient cells

Lactobacillus arabinosus 17–5 was grown as described by McIlwain & Hughes (1948) on the semi-synthetic medium consisting of casein hydrolysate, salts and vitamin supplement. Satisfactory yields (10–15 mg. dry wt./100 ml.) of deficient cells were obtained after 30–44 hr. growth on the medium containing from 1·5 to $2·0 \times 10^{-7}$ M-nicotinic acid (Table 1). The addition of nicotinic acid to glycolysing suspensions of these deficient cells stimulated their rate of glycolysis from two- to three-fold. Higher yields of deficient cells (30–40 mg. dry wt./100 ml.) were obtained after 60–70 hr. growth on the medium containing more nicotinic acid (10^{-6} M), but these cells were unsatisfactory because their rate of glycolysis tended to fall on incubation and the ability to synthesize cozymase was poor. Non-deficient cells were grown on a medium made from enzymically hydrolysed casein, yeast extract and glucose (Nossal, 1951).

Table 1. *The effect of nicotinic acid upon glycolysis by washed suspensions of* Lactobacillus arabinosus 17–5

(The cells were grown as indicated below, collected, washed as described and the effect of nicotinic acid on glycolysis measured manometrically. In each Warburg flask was 2·0 ml. containing: 0·05 M-$NaHCO_3$; 0·05 M-glucose; 2·0–4·0 mg. dry wt. cells. Nicotinic acid (5×10^{-5} M final) was added from the side arm.)

Growth period (hr.)	Nicotinic acid in growth medium (M $\times 10^{-7}$)	Yield of cells (mg. dry wt./100 ml.)	CO_2 (μmoles/mg. dry wt./hr.) Without nicotinic acid	With nicotinic acid
19	100	30	11·0	11·0
19	10	30	10·0	10·0
60	10	45	3·7	6·0
22	5·0	12	17·0	17·0
40	5·0	24	3·0	10·0
21	2·0	15	7·2	11·0
40	2·0	16	3·6	10·8
22	1·0	9	12·0	20·0
40	1·0	10	5·0	15·0
20	0·4	35	10·0	20·0

Measurements of the effects of nicotinic acid on glycolysis and cozymase synthesis

In the main compartment of a conical Warburg cup was placed 0·1 M-glucose, 0·5 ml.; 0·2 M-$NaHCO_3$, 0·5 ml.; washed cell suspension, 0·5 ml. and sufficient Krebs & Henseleit saline to bring the final volume to 2·0 ml. The nicotinic acid or derivative was in the side arm in 0·2 ml. water. Yellow phosphorus was placed in the centre well. The cup was gassed with 95% N_2–5% CO_2, and shaken in a thermostat at 40·0°. Readings were taken at 5 min. intervals until the rate of evolution of CO_2 was constant (20–30 min.). The side arm was then emptied and readings continued at 3–5 min. intervals. To stop the reaction, the cup was taken from the thermostat and placed in an ice-salt freezing mixture. The contents were then allowed to thaw, transferred to a centrifuge tube and centrifuged for 10 min. at approx. 3500 *g*. The supernatant was poured off into a test tube. In order to collect the cells quantitatively the cup was washed out twice with 1·0 ml. 0·9% NaCl, the washings were added to the centrifuged cells and recentrifuged. The washings were discarded and the cells washed once again with 2·0 ml. of 0·9% saline, and then resuspended in 1·0 ml. of 0·065 M-phosphate buffer, pH 6·2, and heated at 80° for 15 min. As shown previously (McIlwain & Hughes, 1948) this treatment releases the cozymase from the cells with a 5–8% loss; no corrections for this loss were made. After heating, the tubes were cooled in running tap water and if not used immediately the contents were frozen solid and stored at −14°.

Determination of cozymase

Cozymase was determined manometrically in the apozymase system of Axelrod & Elvehjem (1942), essentially as described by McIlwain & Hughes (1948). The apparent loss of activity by many batches of apozymase upon storing was found to be associated with the formation of acidic material at 2°. This lowered the pH of the apozymase system from 6·2 to 5·6–5·8 and thus reduced its activity. In later experiments where the apozymase was dried over P_2O_5 and stored over a drying agent such as silica gel at −14° the apozymase lost no activity in a month and lost from 10 to 15% after 2 months. Large blank values in some experiments were found to be due to cozymase present in the calcium hexosediphosphate (obtained from Schwartz Laboratories Inc., New York 17, U.S.A.). To remove the cozymase, the calcium hexosediphosphate was dissolved in 1·1 equivalents of HCl and stirred with one-tenth of its weight in norite for 0·5 hr. at 2°. The norite was removed by filtration and 2 equivalents of $Ba(OH)_2$ added. The pH was adjusted to 8·5 and the precipitate of dibarium salt of hexosediphosphate was filtered off, washed with water and ethanol and dried. Barium was removed from small samples immediately before use by the addition of an equivalent of 0·5 N-Na_2SO_4 to an ice-cold solution of the barium salt in 2 N-HCl.

Determination of nicotinic acid, nicotinamide and nicotinamide derivatives

In most of the experiments, nicotinic acid was determined by the microbiological assay method of Barton-Wright (1946). When it was not desired to distinguish between nicotinic acid and amide, samples for assay were

heated in N-HCl as described in a previous paper (McIlwain & Hughes, 1948). To distinguish between the acid and amide, a portion of the sample was treated with sodium hypobromite as described by Atkin, Schultz, Williams & Freys (1943), to destroy the nicotinamide, and another portion was heated with HCl as before. The nicotinic acid in both portions was estimated by microbiological assay and the nicotinamide content of the sample calculated by difference. In some of the later work, nicotinic acid and nicotinamide were estimated by the chemical methods of Chaudhuri & Kodicek (1948) and Hughes (1949). The acetone condensation method of Levitis, Robinson, Rosen, Huff & Perlzweig (1945) was used to determine the total concentration of N^1-substituted nicotinamide derivatives. Fluorescence was estimated in a fluorimeter employing a photomultiplier tube and similar to that described by Lowry (1948). Solutions of quinine sulphate were used as permanent standards.

Cozymase, nicotinamide ribotide and riboside can act as 'V' factor for bacteria (Gingrich & Schlenk, 1944). Activity as 'V' factor was estimated by measuring the growth response of *Haemophilus parainfluenzae* as described by McIlwain & Hughes (1948). National Type Culture Collection, strains no. 4101, no. 4625 and eight locally isolated strains were tested. Where the strain was found to reduce nitrate, tests for nitrite were made according to Hoagland & Ward (1942). It was found that although nitrite was often detected where no growth could be seen, the response of various strains towards nitrate was too unreliable to be used as a quantitative method for estimating 'V' factor.

Chemical determinations

Ammonia was distilled in a modified Parnas apparatus or in Conway units and determined by the method of Russell (1945) or with Nessler's reagent. A modification of the method of Van Slyke was used for the estimation of glucose (see Wheatley, 1947). The ribose and phosphate contents of the nicotinamide nucleotides were determined as described by Schlenk (1943). Lactic acid was estimated by the method of Friedman & Graeser (1933).

Materials

The first of the two specimens of cozymase used as a standard was that described as 'C' by McIlwain & Hughes (1948); it contained 44% coenzyme I (CoI) and no detectable free nicotinic acid or nicotinamide. The second specimen was prepared from brewer's yeast according to LePage (1949), and contained 50–52% CoI when compared with the first specimen in the apozymase system, and approximately 2·0% free nicotinamide, as judged by microbiological assay. Specimens of dihydronicotinamide ribofuranoside (nicotinamide riboside) were made available through the courtesy of Dr L. J. Haynes, Chemistry Department, University of Cambridge. Tests for 'V' factor activity showed that it had one-quarter to one-tenth of the activity of CoI on a molar basis as 'V' factor (Table 2) (see also Haynes & Todd, 1950). In most freshly isolated strains the amounts of riboside needed for visible growth were higher than those needed in the older strains (see Schlenk & Gingrich, 1944). Specimens of nicotinamide glucoside, galactoside and arabinoside were also synthesized by Dr Haynes; they were without 'V'-factor activity for all strains tested (Table 2). Nicotinamide ribose phosphate (nicotinamide ribotide) was prepared from cozymase by the action of potato pyrophosphatase according to Kornberg & Pricer (1949). Nicotinic acid and nicotinamide were commercial specimens which had been recrystallized several times from water-ethanol mixtures.

Table 2. *'V'-factor activity of nicotinamide nucleosides*

(*Haemophilus parainfluenzae* Strain 4101 was grown as described by McIlwain & Hughes (1948) and growth measured photometrically. Nitrite was detected by the method of Hoagland & Ward (1942); + indicating just detectable amounts and + + + + maximum amounts, and 0 no test for nitrite.)

Growth factor added	Concn. of nicotinamide derivative (M × 10^{-7})	Photometer reading at 22 hr.	Nitrite at 22 hr.
None	—	5·2	0
Cozymase	1·3	25	+ + + +
	0·75	20	+ + +
	0·35	13	.
	0·17	6	0
Nicotinamide riboside	20	20	+ + + +
	4	9	+ +
	1	5	+
	0·2	5	+
Nicotinamide galactoside	100	5	0
	10	5	0
Nicotinamide galactoside with cozymase ($0·75 \times 10^{-7}$ M)	100	20	+ +
	10	20	+ +
Nicotinamide arabinoside	100	5	0
	10	5	0
Nicotinamide arabinoside with cozymase ($0·75 \times 10^{-7}$ M)	100	20	+ + + +
	10	20	+ +
Nicotinamide glucoside	100	5	0
	10	5	0
Nicotinamide glucoside with cozymase ($0·75 \times 10^{-7}$ M)	100	20	+ + +
	10	20	+ +

RESULTS

Stimulation of glycolysis by varying concentrations of nicotinic acid

The addition of nicotinic acid in concentrations from 3×10^{-8} to 10^{-4} M to glycolysing washed suspensions of deficient cells caused a gradual increase in the rate of glycolysis (Fig. 1). The magnitude of the increase was greatest at and above $1·0–5·0 \times 10^{-6}$ M; below this concentration the effect decreased and generally could not be detected in concentrations below 3×10^{-8} M (Table 3). The effect reached its maximum after a lag period of 10–50 min. This lag period was shortest at nicotinic acid concentrations above 5×10^{-5} M, and increased as the concentration was reduced to 10^{-7} M (Fig. 1). This stimulating effect of nicotinic acid is due to the formation of cozymase which in the deficient cells limits glycolysis (McIlwain *et al.* 1949). Thus the lag period may

be taken as indicating the time taken for the cells to synthesize sufficient cozymase to saturate the glycolytic system and hence indirectly indicates the rate of cozymase synthesis. It would therefore appear that below 5×10^{-5} M the concentration of nicotinic acid is a limiting factor in cozymase synthesis. When limiting amounts of nicotinic acid

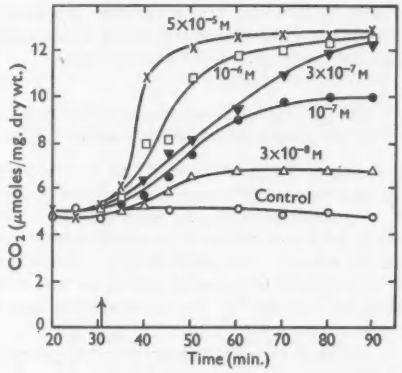

Fig. 1. The effect of varying concentrations of nicotinic acid on glycolysis by deficient cells. Cells were grown for 41 hr. on medium containing $1{\cdot}57 \times 10^{-7}$ M-nicotinic acid and collected and washed as described. Glycolysis was followed manometrically. In each Warburg cup were 2 ml. containing 0·05 M-glucose, 0·05 M-$NaHCO_3$, 3·2 mg. dry wt. of cells. Nicotinic acid added from the side arm as indicated by the arrow.

Table 3. *The effect of varying concentrations of nicotinic acid on glycolysis by deficient cells*

(Cells were grown for 40 hr. on a medium containing $1{\cdot}5 \times 10^{-7}$ M-nicotinic acid. Glycolysis was followed manometrically. In each Warburg flask was 2·0 ml. containing: 0·05 M-glucose; 0·05 M-$NaHCO_3$; 2·3 mg. dry wt. of cells: the nicotinic acid was added from the side arm. The values for CO_2 production are the maximum that occurred during 2 hr. after adding the nicotinic acid.)

Concn. of nicotinic acid ($M \times 10^{-8}$)	CO_2 (μmoles/mg. dry wt. cells/hr.)
None	7·2
3·0	7·8
10·0	9·7
30·0	17·7
100	18·0
150	19·3
500	19·0

are added to the cells, all the nicotinic acid is taken up and converted into cozymase (see p. 335). A limiting concentration of 1·0 to $5{\cdot}0 \times 10^{-6}$ M would mean therefore that 1·0–2·0 μm-moles of nicotinic acid/mg. dry wt. are needed to saturate the glycolytic system of the cells.

The rate of cozymase synthesis at varying concentrations of nicotinic acid

Varying concentrations of nicotinic acid ranging from 5×10^{-4} to 3×10^{-7} M were added to glycolysing deficient cells. The cups were placed in a freezing

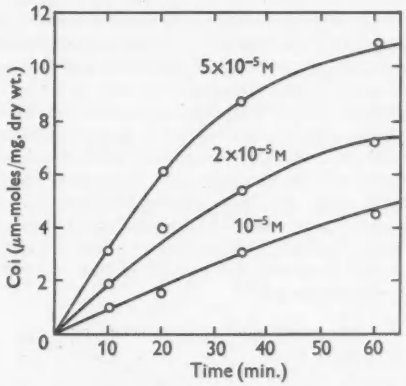

Fig. 2. Cozymase synthesis in varying concentrations of nicotinic acid. Cells were grown for 21 hr. on medium containing 2×10^{-7} M-nicotinic acid, collected and washed. Reaction with nicotinic acid, in concentrations as indicated on the graphs, and cozymase determinations were as described in the text.

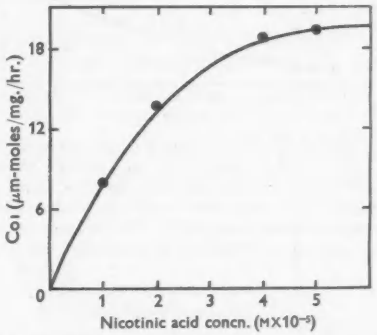

Fig. 3. The initial rate of cozymase synthesis in varying concentrations of nicotinic acid. Cells were grown for 21 hr. on medium containing 2×10^{-7} M-nicotinic acid. The reaction with nicotinic acid was for 10 min. at 40°. Cozymase estimations were carried out as described in the text.

mixture to stop the reaction at 5, 10, 15 and 30 min., the cells collected, cozymase extracted, and estimated as already described. Under these conditions no measurable amounts of cozymase appeared in the reaction fluid and it is therefore assumed that the rate of cozymase accumulation in the cells indicates

the rate of cozymase synthesis. As will be seen from Fig. 2 the cozymase content of the cells rapidly rises following the addition of nicotinic acid. At the higher concentrations of nicotinic acid, the maximum rate of cozymase synthesis lasted for approximately 20–30 min. then decreased and ceased when the cells contained 12–14 μm-moles/mg. dry wt. (Fig. 2). At the lower concentrations of nicotinic acid the rate of cozymase synthesis continued approximately linearly until all the available nicotinic acid was converted into the coenzyme (see also p. 335). The maximum rate of cozymase synthesis (16–25 μm-moles/mg. dry wt./hr.) occurred in concentrations at and above 5×10^{-5} M-nicotinic acid and fell off progressively as nicotinic acid concentration was reduced (Fig. 3). These results confirm the conclusions drawn from the experiments in the preceding paragraph, which suggested that the rate of cozymase synthesis was limited by the concentration of nicotinic acid.

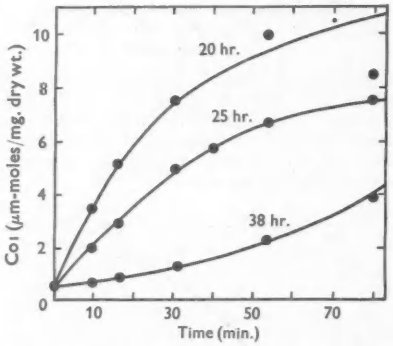

Fig. 4. The effect of growth period on the rate of cozymase synthesis. Cells were grown in medium containing 2×10^{-7} M-nicotinic acid and samples taken at the times indicated. The cells were collected, washed and the reaction with nicotinic acid (5×10^{-5} M final) and cozymase estimations carried out as described.

The effect of growth period upon the cozymase synthesis

The maximum rate of cozymase synthesis, at and above 5×10^{-5} M-nicotinic acid, was found in washed cells grown for 16–20 hr. In three batches of cells grown for a shorter period (10 hr.) the rate of synthesis was 15–20 % lower than maximum. The rate of cozymase synthesis fell progressively as growth was extended from 20 to 70 hr. (Fig. 4). At 38 hr. the initial rate was 1·5–2·0 μm-moles/mg. dry wt./hr. and increased to 6·0–10·0 μm-moles/mg. dry wt./hr. after 50–90 min. In cells grown for 70 hr., however, the rate of cozymase synthesis remained low (0·5–1·0 μm-mole/mg. dry wt./hr.) on incubation up to 3 hr. During this period the rate of glycolysis increased from 1 μmole/mg. carbon dioxide/mg. dry wt./hr. to 4 μmoles/mg. dry wt./hr. Younger cells with an initial rate of glycolysis of from 2 to 5 μmoles carbon dioxide/mg. dry wt./hr. were found to synthesize cozymase at rates of 2–4 μm-moles/mg. dry wt./hr., that is at a rate two- to three-fold faster than in the old cells whose rate of glycolysis was the same. Although, as is shown later, glycolysis is essential for cozymase synthesis, it would appear that the synthesis in older cells is limited by processes other than glycolysis.

The effect of replacing nicotinic acid by nicotinamide and its derivatives

In young deficient cells (19–22 hr. growth) there was no significant difference in the effects on glycolysis or on the rate of cozymase synthesis when nicotinic acid was replaced by nicotinamide, nicotinamide riboside or nicotinamide ribotide. In older cells (30–48 hr. growth) glycolysis was stimulated more rapidly by the nicotinamide and its

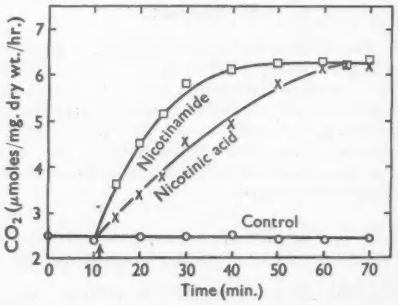

Fig. 5. Stimulation of glycolysis in deficient cells grown for 50 hr., by nicotinic acid and nicotinamide. Glycolysis and reaction with nicotinic acid and nicotinamide (5×10^{-5} M final) were measured manometrically as described in the text.

derivatives than by nicotinic acid, although the final rate of glycolysis was the same (Fig. 5). This suggests that in older cells cozymase is synthesized more rapidly from nicotinamide and its derivatives than from nicotinic acid. Experiments in which the cozymase content of the cells was measured showed no differences in the rate of cozymase synthesis. As shown previously the differences in the amount of cozymase which changed the rate of glycolysis were of the order of 1·0–2·0 μm-moles/mg. dry wt. Differences in the rate of formation of such small amounts of cozymase were too small to be measured by the present methods. No cozymase was synthesized from nicotinamide arabinoside, nicotinamide glucoside or nicotinamide galactoside. This

result agrees with the finding that these compounds are inactive as 'V' factor for *Haem. parainfluenzae*.

Under a variety of conditions the rate of cozymase accumulation in deficient cells was, in the presence of cozymase, 10–15% faster than the accumulation in the presence of nicotinic acid or nicotinamide. Cozymase was not degraded by the organism at rates faster than 0·01 μm-mole/mg. dry wt./hr. (see also McIlwain & Hughes, 1948). The intact cozymase molecule thus appears to be readily adsorbed.

and no nicotinamide or other possible intermediates in the synthesis of cozymase accumulated once the saturation level was attained (Table 5). When experiments were continued for longer periods, i.e. up to 4 hr. instead of the usual 1–2 hr., a small amount of an N^1-substituted nicotinamide compound was detected in the medium. This material had 'V'-factor activity for *Haem. parainfluenzae*, but the amounts as judged by the fluorimetric method of Levitis *et al.* (1945) were equivalent to 0·02 μm-mole

Table 4. *Cellular concentration of nicotinic acid, nicotinamide or cozymase during synthesis by deficient cells*

(Cells were grown on medium as described below, collected and washed. The reaction with nicotinic acid (5×10^{-5} M) and estimation of the products was carried out as described in the text.)

	Time after adding nicotinic acid (min.)	Total nicotinic acid (μmoles/mg. dry wt.)	Nicotinamide (μmoles/mg. dry wt.)	Cozymase (μmoles/mg. dry wt.)
Exp. A. Cells were grown for 28 hr. on medium containing 2×10^{-7} M-nicotinic acid	0	0·5	—	0·46
	10	3·6	—	3·8
	20	6·3	—	6·3
	40	11·1	—	10·8
	80	15·6	—	14·0
Exp. B. Cells were grown for 44 hr. on medium containing $1·57 \times 10^{-7}$ M-nicotinic acid	0	1·2	1·0	1·0
	30	1·8	1·2	1·4
	60	5·0	3·0	3·2

Cozymase was not taken up in the absence of glucose; some source of energy is therefore needed for cozymase adsorption as well as for its synthesis. The highest concentration of cozymase found in cells was the same (10–12 μm-moles/mg. dry wt.) whether nicotinic acid, nicotinamide or cozymase were added to glycolysing deficient cells. Cells grown with adequate nicotinic acid (10^{-4} M) on the semi-synthetic medium or on the yeast-supplemented pronutrin medium also contained the same amount of cozymase and did not adsorb further cozymase. This amount of cozymase (10–12 μm-moles/mg. dry wt.) therefore appears to be a saturation level for this organism and once it is attained the organism ceases either to adsorb further cozymase from the medium or to destroy it as in the case of other micro-organisms (McIlwain & Hughes, 1948).

The metabolism of nicotinic acid

In the absence of glucose, nicotinic acid was not removed from the medium by washed suspensions of cells which had been grown under a variety of conditions. In the presence of glucose, however, nicotinic acid was taken up by deficient cells at approximately the same rate as cozymase was synthesized (Table 4). During synthesis, small amounts of free nicotinic acid sometimes appeared in the cells, but when the cozymase content of the cells had reached 10–12 μm-moles/mg. dry wt. cozymase synthesis and nicotinic acid metabolism ceased. When the cells were provided with up to tenfold excess of nicotinic acid the excess acid was recovered from the medium of cozymase/2·0 ml. and were too small to be estimated in the apozymase system. It is uncertain, therefore, whether the substance leaking from the cells is cozymase or some other N^1-substituted nicotinamide derivative.

Table 5. *Recovery of nicotinic acid from medium and cells after glycolysis*

(Nicotinic acid was added to glycolysing cells (3·0 mg./2 ml.) under the conditions described in Tables 2 and 3. After reacting for 90 min. the cells were collected by centrifugation, washed twice with 0·9% NaCl, and nicotinic acid was estimated in cells and medium by microbiological assay.)

Nicotinic acid added to the medium (μmoles)	Nicotinic acid recovered (μmoles)	
	Cells	Medium
None	2·0	None
6·0	8·0	None
20	16	1·5
50	19	3·1
100	23	80
200	25	180

The metabolism of nicotinamide

Nicotinamide added to glycolysing cells disappeared rapidly from the medium as judged by the fluorescence after reaction with CNBr (Chaudhuri & Kodicek, 1948) (Table 6). Some of the nicotinamide (20–40 μmoles) which disappeared from the medium was accounted for by the appearance in the cells of cozymase, but the rest could not be accounted

for by the accumulation of other derivatives of nicotinic acid or nicotinamide (Table 6). After reaction with nicotinamide the mother liquor was examined by the method of Hughes (1949) (reaction with CNBr at pH 5·5, followed by sodium hydroxide), and the absorption spectrum was found to resemble that of nicotinic acid (Fig. 6). The formation of nicotinic acid from nicotinamide was confirmed by microbiological assay (Table 6). It was also found that there was some temporary increase in the ammonium content of the medium during reaction with nicotinamide by glycolysing cells under these conditions (Table 6). The amount of ammonia expected to be formed under the usual conditions was too small to be accurately estimated since blank values on the cells themselves were sometimes as large as that expected to be formed from the nicotinamide (100 μm-moles). However, ammonia formation at the rate of approximately 2 μmoles/mg. dry wt./hr. was found when cells were incubated with larger amounts of nicotinamide than usual ($2{\cdot}0 \times 10^{-3}$ M). A similar formation of ammonia occurred in the absence of glucose (Table 7). It is clear, therefore, that these organisms deamidate nicotinamide and that this reaction, unlike the uptake of nicotinic acid and the synthesis of cozymase, is independent of the presence of glucose.

Table 6. *Deamination of nicotinamide during cozymase synthesis*

(The reaction was carried out as described in the text. In each Warburg flask were 2·0 ml. containing: 0·05 M-glucose; 0·05 M-$NaHCO_3$; 2·3 mg. dry wt. cells. Nicotinamide (100 μm-moles) was added from the side arm.)

Time after adding nicotinamide (min.)	Relative fluorescence	Nicotinic acid in mother liquor (μm-moles/2·0 ml.)	NH_3 content of mother liquor (μm-moles/2·0 ml.)	Cozymase content of cells (μm-moles)
0	100	0	57	1·2
8	2·7	71	75	8·2
15	*Negative reading	84	87	12·0
30	*Negative reading	85	54	19·0
60	*Negative reading	80	50	19·3

* Negative reading indicates some absorption of the activating light probably by nicotinic acid (cf. Chaudhuri & Kodicek, 1948).

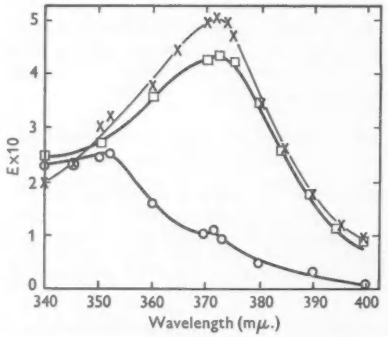

Fig. 6. The change in the absorption curve of nicotinamide brought about by *Lb. arabinosus*. Nicotinamide (100 μm-moles/2 ml.) was added to glycolysing cells under the usual conditions and the reaction stopped at 0 and 10 min. The cells were removed by centrifugation, 1·0 ml. of the mother liquors reacted with CNBr and NaOH and the absorption spectra measured in a Beckman spectrophotometer. ○—○, solution at 0 min. The absorption spectra is typical of nicotinamide ($E_{max.}$ at 352 mμ.). □—□, solution at 10 min. ($E_{max.}$ at 372 mμ.). ×—×, control solution of nicotinic acid ($E_{max.}$ at 373 mμ.).

Table 7. *Nicotinic acid and* NH_3 *formation from nicotinamide in the absence of glucose*

(*Lb. arabinosus* was grown on the Pronutrin medium for 16 hr., collected by centrifugation and washed in 0·9% NaCl. Reaction was carried out in double side-arm test tubes (Hughes & Williamson, 1952). In each was 10·0 ml. 0·2 M-phosphate buffer, pH 6·5; 0·002 M-nicotinamide; and 5 mg. dry wt. cells. The tubes were incubated at 40° and the reaction stopped by the addition of 1 ml. 2 N-H_2SO_4. Ammonia was estimated after distillation and nesslerization. Nicotinic acid was estimated by microbiological assay after destruction of the remaining nicotinamide.)

Time after mixing (min.)	NH_3 formed (μmoles)	Nicotinic acid formed (μmoles)
0	0·91	1·2
15	2·1	2·3
45	3·8	4·3
90	7·6	8·2
180	13·2	13·8

The maximum rate of deamidation of nicotinamide (1–2 μmoles/mg. dry wt./hr.) was some 200 times faster than the maximum rate of cozymase synthesis and appeared to be independent of the time for which the cells had been grown (up to 4 days). Cells grown on the semi-synthetic medium with adequate nicotinic acid and cells grown on the 'pronutrin' medium also deamidated nicotinamide at the same rate as the deficient cells. The rate of deamidation is therefore independent of the

cozymase content of the cells. In cells grown for 40–50 hr. the initial rate of cozymase synthesis from nicotinamide was 1·5–4·0 μm-moles/mg. dry wt./hr. Under these conditions all added nicotinamide (100 μm-moles) was converted to nicotinic acid in the first 5–8 min. of the reaction. This means that the bulk of the cozymase synthesized by the cells after 2 hr. (8–10 μm-moles/mg. dry wt.) could not have been directly derived from the added nicotinamide.

The effect of constituents of the growth medium on cozymase synthesis by washed suspensions

To the washed suspensions, the constituents of the growth medium were added as shown in Table 8 under the conditions described for measuring

Table 8. *The effect of the constituents of the growth medium on the stimulation of glycolysis by nicotinic acid in washed suspensions*

(Glycolysis was followed manometrically. In the main compartment of each Warburg vessel was 2·0 ml. containing 0·02 M-glucose, 0·02 M-$NaHCO_3$, 2·3 mg. dry wt. cells, and the substance tested in the final concentration shown below. Nicotinic acid (5×10^{-5} M final) was added from the side arm.)

Substance added	CO_2 (μmoles/mg. dry wt./hr.) Without nicotinic acid	With nicotinic acid
None	4·0	12·0
Acid casein hydrolysate (1·0%)	3·9	10·7
Tryptophan 0·04%, cystine 0·04%	4·0	12·0
Vitamin mixture (Barton-Wright, 1946)	4·0	12·0
Adenine, uracil, xanthine (0·02 mg. of each)	4·0	12·0
Guanine (0·02 mg.)	4·8	12·8
Glutamine (0·01 M)	3·8	11·4
Ribose (0·1 M)	3·8	11·4
NH_4Cl 0·01 M and salts B (Barton-Wright, 1946) 0·01 ml.	4·3	12·9

glycolysis. With the possible exception of guanine none of the constituents of the growth medium had any effect on the nicotinic stimulation of glycolysis. In some experiments guanine markedly decreased the length of the lag period. This effect could not be repeated at will in all batches of otherwise identical deficient cells and the effect was not investigated further. The rate of cozymase synthesis was not affected by addition of the vitamin mixture, tryptophan, glutamine, guanine, ribose, ammonium chloride, and casein hydrolysate. The omission of glucose completely stopped the synthesis of cozymase from nicotinic acid, nicotinamide and nicotinamide nucleotide. Neither pyruvic acid nor adenosinetriphosphate replaced glucose, but no other sources of energy were tested. In the presence of glucose the rate of cozymase synthesis from nicotinic acid derivatives was the same when the reaction was carried out in air instead of in nitrogen, and when the bicarbonate was replaced by 0·05 M-phosphate buffer, pH 7·8. Estimation of the glucose and lactic acid showed that the rates of glycolysis in air and in nitrogen with phosphate and bicarbonate media were identical. The rate of cozymase synthesis was not significantly altered by changes in the pH of the phosphate buffer from pH 5·8 to 8·2.

DISCUSSION

Catalytic effect of CO1. The catalytic effect of cozymase, expressed as a turnover number, i.e. mol. CO1/mol. acid formed/hr. (McIlwain, 1949) was 0·5 to $3·0 \times 10^4$ in washed suspensions of deficient cells. In cells supplemented with nicotinic acid, although the rate of glycolysis increased from two- to four-fold, the catalytic effect of cozymase remained the same. The value found for cozymase in washed suspensions agrees with the turnover number of nicotinic acid during growth under the conditions of the microbiological assay (McIlwain *et al.* 1949). This agreement indicates that deficiency in nicotinic acid does not greatly affect processes such as the synthesis of enzymes concerned in the glycolytic system as was found to be the case with biotin (Blanchard, Korkes, del Campillo & Ochoa, 1950). The catalytic effect of cozymase in cells grown upon medium containing an excess nicotinic acid, or in washed cells which had made cozymase from nicotinic acid was $3·0 \times 10^3$, i.e. about ten times lower than in the deficient cells. The cells are therefore able to synthesize and bind about ten times more cozymase than is needed for glycolysis. The saturation level of cozymase (10–12 μm-moles/mg. dry wt.) appeared not to be limited by supplies of the other parts of the cozymase molecule, ribose, adenine or phosphate, nor by constituents of the growth medium other than glucose.

Route of cozymase synthesis. In most species of bacteria, nicotinamide is as readily or more readily available for growth as is nicotinic acid (Knight, 1945). These findings are generally believed to indicate that the acid is amidated as the first step in the synthesis of cozymase. This idea is also supported by the studies on the inhibitory effects of pyridinesulphonic acid and pyridinesulphonamide (McIlwain, 1940) and by the present studies on the stimulation of glycolysis by nicotinamide and nicotinic acid in which the amide appeared to be more readily available than the acid. Further studies on the metabolism of nicotinamide showed, however, that even in young cells, where the synthesis of cozymase was most rapid, nicotinamide was deamidated some 50 to 100 times faster than cozymase was synthesized. In fact, in the older cells very little cozymase could have been synthesized from the

added nicotinamide and was made instead from the nicotinic acid formed by deamidation of the amide. But this does not rule out the possibility that nicotinamide is an intermediate in the synthesis of cozymase since reactions causing the breakdown of essential metabolites are of common occurrence in bacteria. For instance the breakdown of glutamine has been shown to occur in *Clostridium welchii* (Krebs, 1948; Hughes & Williamson, 1952) at a much faster rate and by a different reaction than it is synthesized (Fry, 1949). In erythrocytes, while nicotinic acid readily gives rise to cozymase, no cozymase was formed from nicotinamide (Handler & Kohn, 1943; Hoagland & Ward, 1942). In certain strains of *Pasteurella* and *Leuconostoc* nicotinic acid but not nicotinamide supports growth (Johnson, 1945; Koser & Kasai, 1950). These results could be taken as indicating that nicotinamide is not an intermediate in cozymase synthesis. The evidence as to whether nicotinic acid is amidated to form nicotinamide or whether nicotinic acid riboside is formed first and is then amidated must therefore remain uncertain. *Lb. arabinosus* does not seem to be suitable material on which to study this question, since no intermediates in the synthesis of cozymase from nicotinic acid accumulate during synthesis and excess nicotinamide is decomposed by the organism.

SUMMARY

1. Glycolysing washed suspensions of cozymase-deficient cells of *Lactobacillus arabinosus* 17–5 synthesized cozymase from nicotinic acid, nicotinamide riboside and nicotinamide ribotide.

2. The newly formed cozymase stimulated the rate of glycolysis by the cells from two- to five-fold. Maximum stimulation occurred in the presence of 1 to $5{\cdot}0 \times 10^{-6}$ M-nicotinic acid.

3. As judged by their effects on glycolysis, nicotinamide, nicotinamide riboside and ribotide were in older cells more readily available for cozymase synthesis than was nicotinic acid.

4. The rate of cozymase synthesis in any batch of cells was dependent on the added nicotinic acid concentration and was maximal at and above 5×10^{-5} M-nicotinic acid.

5. The maximum rate of cozymase synthesis (18–24 μm-moles/mg. dry wt./hr.) occurred in cells grown for 18–24 hr. and fell progressively as the cells were grown for longer periods.

6. Cozymase synthesis and the uptake of nicotinic acid ceased when the cells contained from 10 to 12 μm-moles cozymase/mg. dry wt.

7. Cozymase uptake and synthesis and the uptake of nicotinic acid and nicotinamide did not occur in the absence of glucose. No other constituent of the growth medium had marked effect on these processes.

8. Nicotinamide was deamidated rapidly (1–2 μmoles/mg. dry wt./hr.). This reaction was independent of the presence of glucose and of the cozymase content of the cells.

The authors wish to express their gratitude to Prof. H. A. Krebs, F.R.S., for his advice and criticism. Thanks are due to Dr L. J. Haynes for gifts of synthetic nicotinamide derivatives.

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Studies in Detoxication

44. THE METABOLISM OF BENZENE. THE MUCONIC ACID EXCRETED BY RABBITS RECEIVING BENZENE. DETERMINATION OF THE ISOMERIC MUCONIC ACIDS

By D. V. PARKE and R. T. WILLIAMS
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 1

(*Received* 16 *October* 1951)

Muconic acid (hexa-2:4-diene-1:6-dioic acid) was first discovered to be a metabolite of benzene by Jaffé (1909), who isolated it from the urine of dogs and rabbits receiving benzene. There are three geometrical isomers of muconic acid, namely, *cis-cis*, *cis-trans* and *trans-trans*, and in every instance in which the isolation of muconic acid from the urine of men and animals dosed with benzene has been reported, the isomer isolated has been the *trans-trans* form (Jaffé, 1909; Fuchs & Soós, 1916–17; Mori, 1918; Neumaerker, 1923; Thierfelder & Klenk, 1924; Drummond & Finar, 1938; Bernhard & Gressly, 1941). If muconic acid is formed *in vivo* by the opening of the benzene ring, then it would be expected on stereochemical grounds that the isomer produced would be the *cis-cis*-isomer; the oxidation of phenol or of catechol with peracetic acid does, in fact, give rise to *cis-cis*-muconic acid (Böeseken & Engelberts, 1931; Böeseken & Kerkhoven, 1932; Elvidge *et al.* 1950*a*).

The isolation of the *trans-trans*-acid from benzene urine, however, raised doubt as to whether benzene was the source of the acid, until Bernhard & Gressly (1941) showed that benzene containing deuterium gave rise *in vivo* to muconic acid containing deuterium. The *trans-trans*-acid could, however, arise from the *cis-cis*-acid by isomerization during the isolation procedures, but Drummond & Finar (1938) showed that this was probably not the case and they suggested that the isomerization probably took place *in vivo*.

That *cis-cis*-muconic acid is probably the first muconic acid to be derived biologically from benzene is suggested by the observation of Hayaishi & Hashimoto (1950) that an enzyme called pyrocatecase separated from a strain of *Pseudomonas* sp. oxidizes catechol to what they suggest is *cis-cis*-muconic acid, which was isolated in high yield in the crystalline state. Catechol is known to be a metabolite of benzene in the rabbit (Porteous & Williams, 1949*a*, *b*).

Recently, however, Elvidge, Linstead, Sims & Orkin (1950*b*) identified, for the first time, the third isomer of muconic acid, namely, the *cis-trans*-isomer, and they showed that recrystallization of the *cis-cis*-acid from water (but not ethanol) was sufficient to convert it into the *cis-trans*-acid. This observation thus casts doubt on much of the earlier work which involved the use of the so-called *cis-cis*-acid.

The objects of the present work were to find methods of estimating and distinguishing between the three isomers of muconic acid, and to find out whether or not the urinary muconic acid was a mixture of isomers, since earlier workers could have lost the more soluble *cis*-isomers during purification. We shall show, however, that all the muconic acid of benzene urine from rabbits is almost certain to be the *trans-trans*-isomer.

EXPERIMENTAL

Preparation of materials

Melting points are uncorrected. *cis-cis*-Muconic acid was readily prepared by the oxidation of phenol by concentrated peracetic acid. (Elvidge *et al.* (1950*a*) used dilute peracetic acid, but in our hands better results were obtained with the concentrated acid.) The peracetic acid was prepared by cautiously mixing in the cold 10 ml. of 70% (w/w) H_2O_2 with 19 ml. of acetic anhydride, the molecular ratio of H_2O_2 to Ac_2O being 2:1·5, and keeping in cold water for 18 hr. Phenol (3 g.) was then added with shaking until it dissolved, and the mixture was kept in the dark. After 24 hr. the crystalline deposit of the *cis-cis*-acid was collected on a sintered-glass filter. Further quantities were obtained and filtered off each day by keeping the solution for 3 more days. The yield was 1·1 g. or 25%. The combined precipitates were washed with a little ethanol and twice recrystallized from ethanol. It formed colourless rhombs and had m.p. 194° as determined by the method of Elvidge *et al.* (1950*b*); the benzhydryl ester (Elvidge *et al.* 1950*b*) had m.p. 156°.

cis-trans-Muconic acid was prepared by twice recrystallizing the *cis-cis*-acid from hot water according to Elvidge *et al.* (1950*b*). It formed colourless prismatic needles from water, m.p. 190°, and its benzhydryl ester had m.p. 142°. A mixture of the *cis-cis*- and *cis-trans*-acids melted at 179–182°.

trans-trans-Muconic acid formed colourless minute prisms, m.p. 300° (decomp.) and was prepared according to Ingold (1921). The benzhydryl ester had m.p. 190° (cf. Elvidge *et al.* 1950*b*).

The lactones related to muconic acid were prepared by the methods of Elvidge *et al.* (1950*b*). The dilactone, butanolidobutanolide (3:7-diketo-2:6-dioxa*bicyclo*[3:3:0]octane) formed pale brown prisms, m.p. 134° (Elvidge *et al.* (1950*b*) give m.p. 131°), depressed by admixture with the mono-

lactone to 90–95°. The monolactone, γ-carboxymethyl-Δ^{α}-butenolide, formed very pale brown rhombs, m.p. 110° depressed on admixture with the dilactone to 90–95° (Elvidge *et al.* (1950*a*) give m.p. 110·5–111·5°).

The colour reaction between muconic acid and phenol

When *cis-cis-* and *cis-trans*-muconic acids were heated at 130° for 5 min. with various phenols, together with a drop of conc. H_2SO_4, coloured products were obtained which were soluble in water and organic solvents and fluoresced in ultraviolet light. The *cis-trans*-acid gave more intense colours than the *cis-cis*-acid, whereas the *trans-trans*-acid under the same conditions gave no coloured product at all. These observations are summarized in Table 1.

Table 1. *Colours obtained by condensing* cis-cis- *and* cis-trans-*muconic acids with phenols at* 130° *for* 5 *min.*

Condensing phenol	Colour of product: In acid solution	Colour of product: In alkaline solution
Phenol	Crimson*	Blue-green*
Resorcinol	Red-brown*	Red†
Catechol	Brownish violet	Olive green*
Quinol	Brown	Yellow brown‡
1-Naphthol	Brown*	Brown*
2-Naphthol	Brown*	Yellow†

* Light blue fluorescence in ultraviolet light.
† Green fluorescence in visible light.
‡ Blue fluorescence in visible light.

Identification of the muconic acids by the above colour reaction

The phenol most suitable for our purposes was phenol itself. The *cis-cis-* and *cis-trans*-acids give an intense crimson product in 5 min., whereas with the *trans-trans*-acid only a faint pink colour is obtained after several hours' heating. If 1 mg. of *cis-trans*-muconic acid is heated with 500 mg. phenol and 0·1 ml. conc. H_2SO_4 for 10 min. at 130° and the product dissolved in 50 ml. ethanol, this solution gives an extinction ($E_{1\text{ cm.}}$) of 0·7 on the Spekker absorptiometer using Ilford Spectrum green filter no. 604 and heat filter H 503. *trans-trans*-Muconic acid under the same conditions gives $E_{1\text{ cm.}} = 0{\cdot}01$. With *cis-cis*-muconic acid the maximum colour is attained in 45 min. when $E_{1\text{ cm.}} = 0{\cdot}76$, whereas with *trans-trans*-muconic acid in 45 min. $E_{1\text{ cm.}}$ is only 0·05.

If the temperature of the reaction is raised above 140°, *trans-trans*-muconic acid gives the same crimson colour as its isomers, maximum colour being attained in 20 min. at 160°. At 100°, the *cis-cis-* and *cis-trans*-acids give crimson colours after several hours' heating, but the *trans-trans*-acid gives no colour at all at this temperature. Thus by carrying out the test at different temperatures the *trans-trans*-acid can readily be distinguished from its isomers.

The two lactones, γ-carboxymethyl-Δ^{α}-butenolide and butanolido-butanolide, give the same colour at the same rate as *cis-trans*-muconic acid, and it is possible that one of these lactones is the precursor of the colour, although Elvidge *et al.* (1950*a*) were unable to convert the *trans-trans*-acid into these lactones. Elvidge *et al.*, however, point out that when the *trans-trans*-acid is heated at 160–165° with 95% H_2SO_4, none can be recovered.

Quantitative estimation of muconic acid

The above colour reaction can be used for the quantitative estimation of muconic acid and the conditions for its use were investigated as follows.

Quantity of phenol and sulphuric acid. Experiments showed that the optimum amounts of phenol and conc. H_2SO_4 for development of the colour were 500 mg. and 0·1 ml., respectively, to about 1 mg. of muconic acid.

Effect of time of heating. In order to estimate the *cis-cis-* and *cis-trans-* in the presence of the *trans-trans*-acid it became necessary to carry out colour development at 100° for the former two acids and at 160° for the latter. The time of development of maximum colour at these temperatures

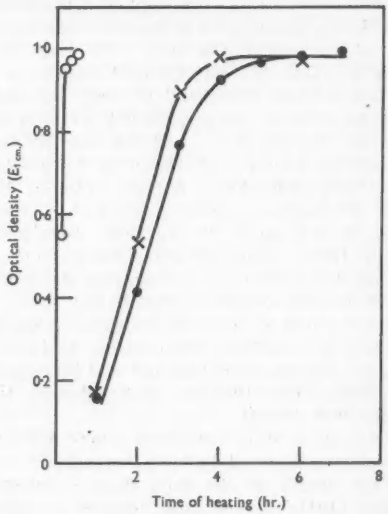


Fig. 1. The effect of time of heating on the production of the red colour obtained by condensing muconic acid (0·5 mg.) with phenol (0·5 g.) and concentrated H_2SO_4 (0·1 ml.) and then dissolving in 50 ml. absolute ethanol. ×—×, *cis-trans*-Muconic acid condensed at 100°; ●—●, *cis-cis*-muconic acid condensed at 100°; ○—○, *trans-trans*-muconic acid condensed at 160°. *E* measured in the Spekker absorptiometer.

had to be determined. Muconic acid (0·5 mg.) was condensed with 0·5 g. phenol and 0·1 ml. conc. H_2SO_4 in corked tubes, at 100° ± 1° for the *cis-cis-* and *cis-trans*-isomers and at 160° ± 2° for the *trans-trans*-acid. Heating was continued for varying lengths of time, and then the coloured melts were dissolved in ethanol to a volume of 50 ml. The colour was measured in 1 cm. cells in the Spekker absorptiometer using colour filters as above. Maximum colour development was attained at 100° in 4 hr. with *cis-trans-* and the two lactones, and in 6 hr. with *cis-cis*-muconic acid. The *trans-trans*-acid gave no colour in 6 hr. at 100°, but gave the same maximum colour in 20 min. at 160° (see Fig. 1) as the *cis*-acids did in 6 hr. at 100°.

Effect of temperature. The above experiments showed that *cis-cis-* and *cis-trans*-muconic acids could be estimated by

heating with phenol and H_2SO_4 at 100° for 6 hr., whereas the *trans-trans*-acid could be estimated in the same way except that the heating had to be done at 160° for 20 min. In order to estimate the *cis*-acids in the presence of the *trans-trans*-acid, it was necessary to find out whether the *cis*-acids gave the same amount of colour at 160° as at 100°. It was found, however, that if the *cis*-acids were condensed at 160°, more colour was produced than at 100°. This was not seen with the *trans-trans*-acid, for temperatures above 160° merely accelerated the destruction of the colour produced by this acid. The effect of temperature on the development of colour with the three acids is shown in Fig. 2. In these experiments colour density was measured at 520 mμ. (see below) in a Unicam spectrophotometer, the instrument finally adopted for all measurements of this colour. This extra colour

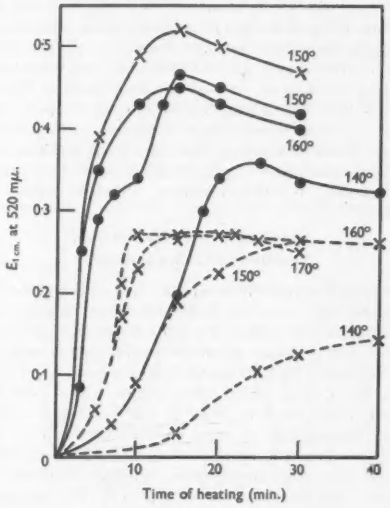


Fig. 2. The effect of temperature on the intensity of the red colour produced by heating the muconic acids with phenol and sulphuric acid. ×– – –×, 0·1 mg. *trans-trans*-acid condensed for varying times at different temperatures and the product dissolved in 50 ml. ethanol; ●—●, 0·1 mg. *cis-cis*-acid treated similarly; ×—×, 0·1 mg. *cis-trans*-acid treated similarly. *E* measured in the Unicam spectrophotometer.

production at 160° with the *cis*-acids, although constant, showed that the *cis*-acids could not be readily estimated in the presence of the *trans-trans*-acid, although if the *trans-trans*-acid occurred alone it could be estimated. In benzene urine only one isomer, the *trans-trans*-, occurred, so that it could be readily estimated by this colour reaction. The elimination of this difficulty is described later (see below).

Stability of the colour. The colour in ethanol when exposed to air was stable for 15 min. after which time it slowly deteriorated probably due to absorption of moisture. *E* values at 0, 0·25, 0·5, 1, 2, 3 and 4 hr. were 0·78, 0·79, 0·75, 0·68, 0·55, 0·49 and 0·44 respectively. All readings were therefore carried out within 15 min. of development. Water (1–2%) reduced the intensity of the colour by a half.

Absorption spectrum of the colour and possible interference by other dicarboxylic acids. It seems possible from the mode of production of the colour, that a xanthone derivative is formed by the condensation of one of the lactones of muconic acid with two molecules of phenol. If this is true, then the possibility that other dicarboxylic acids can form such coloured complexes must be borne in mind especially when the method is applied to urine.

The absorption spectrum of the coloured complex in ethanol in the visible region showed a single maximum at 520 mμ. with $E_{1\,cm.}=0{\cdot}56$ for a solution prepared from 0·2 mg. muconic acid (*cis-cis*- and *cis-trans*- by condensation at 100° and *trans-trans*- at 160°) and made up to 50 ml. with ethanol, measurements now being made with a Unicam spectrophotometer (see Fig. 3 curve *A*). All three isomers condensed as mentioned above gave the same $E_{1\,cm.}$.

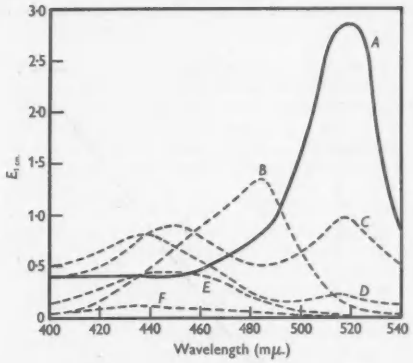


Fig. 3. Absorption spectra of the colours obtained by condensing at 160° for 20 min., certain organic acids with phenol and concentrated H_2SO_4 and dissolving the product in 50 ml. ethanol. Each curve corresponds to 1 mg. of the acid. *A*, *trans-trans*-muconic acid; *B*, oxalic acid; *C*, adipic acid; *D*, pimelic acid; *E*, succinic acid; *F*, suberic acid.

Common acids of the urine were first tested, namely benzoic, hippuric, phenylacetic, salicylic and *p*-hydroxybenzoic acids. When condensed with phenol and sulphuric acid at 160°, these acids give pale pink melts which dissolved with ethanol to give nearly colourless solutions. These acids did not interfere.

The dicarboxylic acids, oxalic, succinic, adipic, pimelic, suberic and azelaic acids were tested and all were found to give coloured melts at 160°, the spectra of which are recorded in Fig. 3. At 520 mμ., the principal interference would come from adipic acid whose coloured complex (formed at 160°) at this wavelength has 33% of the absorption of the phenol condensation product of muconic acid. In the same way, pimelic and oxalic acids would interfere to the extent of 7 and 5% respectively. In the estimation of *cis-cis*- and *cis-trans*-muconic acids, that is with a condensation temperature of 100°, the interference would be much less being 1% for oxalic, 0·9% for adipic and 0·4% for pimelic acid.

The amounts of these dicarboxylic acids in rabbit urine are likely to be very small, although values are known only

for human urine. Hanson (1943) has reported traces of succinic, adipic and suberic acids in normal human urine, and gives the content of $COOH(CH_2)_nCOOH$ as 15 mg./l. The succinic acid content of normal human urine has been reported by Weitzel (1947) as being 1·9–8·8 mg./l. in twelve subjects. According to Mori (1918) rabbits excrete about 4–9 mg./day of oxalic acid. The amounts of these dicarboxylic acids excreted, however, depend upon diet (Hanson, 1943; Weitzel, 1947).

Recovery of muconic acid from aqueous solutions. Solutions of *cis-cis-* or *trans-trans*-muconic acids (0·5 or 0·2 mg.) in 0·1 N-Na_2CO_3 (1 ml.) were treated with 2 N-HCl (0·05 ml.), and the solutions were evaporated at 105°. Phenol and H_2SO_4 were then added as above, and the tubes containing the *cis-cis*-acid heated for 6 hr. at 100° and those containing the *trans-trans*-acid at 160° for 20 min. The colour was measured as before. Recoveries were satisfactory (95–102%). The standard curves for the three acids are shown in Fig. 4.

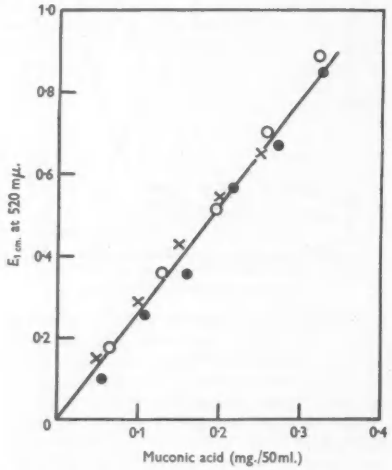


Fig. 4. Standard curve for the estimation of muconic acid, prepared by condensing muconic acid with phenol (0·5 g.) and conc. H_2SO_4 (0·1 ml.) at 100° for 6 hr. with the *cis-cis-* and *cis-trans*-acids and at 160° for 20 min. with the *trans-trans*-acid and then dissolving in 50 ml. absolute ethanol. $E_{1\,cm.}$ at 520 mμ. measured in the Unicam spectrophotometer. ×, *cis-cis*; ●, *cis-trans*; ○, *trans-trans*.

Estimation of muconic acid in urine. The above method was now applied to rabbit urine. If this urine were dried at 105°, subsequent treatment with phenol and H_2SO_4 yielded dark-brown solutions. Much of this darkening could be avoided if the urines were dried at room temperature *in vacuo* in a desiccator over P_2O_5. This also had the advantages of avoiding heat (which might cause isomerization), treatment with organic solvents and non-quantitative extraction procedures.

Normal urines from rabbits were collected in flasks containing 0·5 ml. 1% $HgCl_2$. Each 24 hr. urine (slightly less than 100 ml.) was diluted with water to 100 ml. Solutions of *cis-cis-* and *trans-trans*-muconic acids (5 mg./ml. in N-$NaHCO_3$) were added to 25 ml. of these urines which were then diluted with water so that the final concentration of muconic acid in each solution was 0·1 mg./ml. In this way, solutions of muconic acid corresponding to outputs of muconic acid of 20 and 100 mg./day were made up. 1 ml. portions of these diluted urine-muconic acid solutions in test tubes ($3 \times \frac{5}{8}$ in.) were then dried *in vacuo* over P_2O_5 at room temperature. Phenol (0·5 g.) and conc. H_2SO_4 (0·1 ml.) were then added to each dry residue. The tubes containing the *cis-cis*-acid were then heated at 100° for 6 hr. and those containing the *trans-trans*-acid at 160° for 20 min. The product from each tube was then dissolved in 50 ml. ethanol and filtered and the $E_{1\,cm.}$ at 520 mμ. then measured in the spectrophotometer. The recoveries were *cis-cis-* (at 20 mg./day), 78–85% and at 100 mg./day 95–98%; *trans-trans-* (at 20 mg./day), 89–95% and at 100 mg./day, 95–99%.

Recovery of muconic acid from benzene urine. Muconic acid (20 mg. in the least amount of N-$NaHCO_3$) was added to the 24 hr. filtered urine of a rabbit which had received a dose of 0·5 g./kg. of benzene, so that the concentration of added muconic acid was 0·2 mg./ml. Then 1 ml. samples of this urine were dried *in vacuo* and condensed with phenol as above. Blank estimations were carried out simultaneously on the benzene urine. The recoveries of added muconic acid were *cis-cis-*, 102–108; *cis-trans-*, 103–105; *trans-trans-*, 90–97%.

Muconic acid in the urine of rabbits receiving benzene

Benzene was administered orally to rabbits and the urine collected daily, made up to 100 ml. where necessary, and centrifuged. For each urine, eight tubes were set up containing 1 ml. of urine which was evaporated *in vacuo* in a desiccator over P_2O_5. When dry, 500 mg. phenol and 0·1 ml. conc. H_2SO_4 were added to each tube. Three tubes were heated at 100° for 6 hr., three at 160° for 20 min. and two tubes (blanks) kept at room temperature for 6 hr. Each melt was then dissolved in ethanol, made up to 50 ml., filtered from any precipitated inorganic salts and the optical density measured at 520 mμ. in the Unicam spectrophotometer. Normal urines collected before and after the feeding of benzene were treated in the same way. The colour (a general absorption) given by normal urine after heating at 100° for 6 hr. or at 160° for 20 min. remained very constant and gave a reading of $E_{1\,cm.}$ approx. 0·1. In none of the experiments with benzene was there any increase in absorption at 520 mμ. above that of the normal urines after condensation at 100° for 6 hr. From this it was concluded that the urine of rabbits fed benzene does not contain *cis-cis-* and *cis-trans*-muconic acids in amounts greater than 0·05% of the dose of benzene. The figure 0·05% is the limit imposed by the method. The amounts of *trans-trans*-muconic acid found are given in Table 2, which shows that at a dose level of 0·5 g./kg. 0·5% of the benzene appears as *trans-trans*-muconic acid. Fig. 5 shows an individual result.

Metabolism of muconic acid by micro-organisms

Since Evans & Smith (1951) have shown that *cis-cis*-muconic acid, but not the *cis-trans-* and *trans-trans*-isomers, is metabolized by certain micro-organisms, we had to consider the possibility that this acid might be formed and excreted by rabbits receiving benzene and then be rapidly

Table 2. trans-trans-*Muconic acid excretion by rabbits receiving benzene orally*

(*cis-cis-* and *cis-trans-*Muconic acids were absent from all these urines since no colour was obtained on prolonged heating at 100° with phenol and sulphuric acid. Dose level 0·5 g./kg. body wt. except for the two experiments at the foot of the table.)

Rabbit no.	Dose of benzene (g.)	Period of excretion (days)	*trans-trans-* Muconic acid excreted (mg.)	Benzene excreted as *trans-trans-* muconic acid (% of dose)
51	1·58	2	17·6	0·6
53	1·41	1	11·3	0·45
67	1·41	2	12·3	0·5
69	1·23	1	22·3	1·0
70	1·76	3	17·2	0·55
75	1·32*	2	4·9	0·15
82	1·32*	2	9·3	0·4
87	1·32*	1	14·7	0·65
69	2·46	3	17·1	0·4
70	3·52	3	22·0	0·35

* Urine collected in the presence of $HgCl_2$.

destroyed by micro-organisms infecting the urine. We found that the recovery of added *cis-cis-*muconic acid from very stale rabbit urine was negligible. In two experiments we recovered no *cis-cis-*muconic acid after 48 hr. when it was added to stale urine in concentrations of 20 and 100 mg./100 ml., whereas the *trans-trans-*acid could be recovered almost quantitatively.

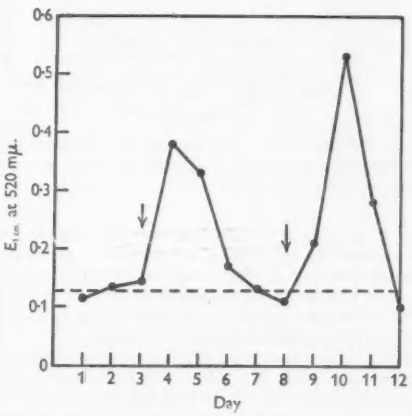


Fig. 5. The excretion of *trans-trans-*muconic acid by rabbit no. 70 which had received orally 1·76 g. of benzene on day 3 and 3·52 g. of benzene on day 8. The muconic acid excretion is expressed in terms of the $E_{1\,cm.}$ at 520 mμ. of the phenol-muconic acid colour in ethanol (see text).

On culturing this urine on McConkey agar two predominant organisms were isolated. One was a late-lactose-fermenting paracolon bacillus and the other a faecal-type streptococcus (enterococcus). These organisms grew very scantily in 48 hr. on synthetic media of salts and vitamins which contained $(NH_4)_2SO_4$ and urea as sources of nitrogen and *cis-cis-*muconic acid as the source of carbon. None of the muconic acid was metabolized. These organisms however grew well on fresh, autoclaved rabbit urine containing *cis-cis-*muconic acid, and in 96 hr. the faecal-type streptococcus metabolized 100% and the paracolon bacillus, 96% of the added *cis-cis-*muconic acid (0·5 mg./ml.). On subculturing 4 or 5 times on glucose nutrient agar media the organisms lost their ability to grow well in rabbit urine containing *cis-cis-*muconic acid.

It was concluded from these observations that in our experiments these organisms did not occur in sufficient numbers and did not metabolize *cis-cis-*muconic acid sufficiently rapidly to affect our results. In any case heavy growth of these organisms only occurred when rabbit urine had been allowed to stand for several days at room temperature. However, in order to eliminate this possibility, the urine from rabbits receiving benzene was collected in flasks containing 0·5 ml. of 1% $HgCl_2$. In this concentration $HgCl_2$ had no effect on the colour development. The results of these experiments (see Table 2) were essentially the same as those in which no $HgCl_2$ was used, and it was concluded that the absence of *cis-cis-*muconic acid was not due to its destruction by micro-organisms.

Isolation of muconic acid from benzene urine

A total of 7 g. of benzene (0·5 g./kg.) was fed by stomach tube with water to four rabbits. The 48 hr. urine (450 ml.) was made 0·2 N with respect to HCl by adding conc. HCl. It was then saturated with $(NH_4)_2SO_4$ and continuously extracted for 6 hr. with peroxide-free ether. (In separate experiments it was shown that 150 mg. of *cis-cis-*muconic acid added to 100 ml. normal rabbit urine could be recovered almost quantitatively by ether extraction for 6 hr. at pH 2, as a muconic acid m.p. 185–186°, probably a mixture of *cis-cis* and *cis-trans*. No *trans-trans-*muconic acid was formed in this procedure.) The ethereal extract was decanted from a small quantity of wet tarry material and allowed to concentrate at room temperature. After cooling at 0° for several hours, the nearly colourless crystals (69 mg. or 0·6% of the dose) which had separated were filtered and washed with a little ether. Evaporation of the mother liquors yielded another 51 mg. of semi-crystalline material.

The colourless crystals were impure, for they began to melt at 180–185° and finally decomposed at 295–300°. On colorimetric assay, as described earlier, they contained no *cis-cis* or *cis-trans-*muconic acid and 20% of *trans-trans-*

muconic. This crude material was also tested microbiologically by Dr W. C. Evans of Aberystwyth (Evans & Smith, 1951) who reports that it contained no *cis-cis*-muconic acid.

Recrystallization of this material from ethanol eventually yielded pure *trans-trans*-muconic acid, m.p. 300° (decomp.); the yield of pure material in several experiments was 0·1–0·2% of the dose of benzene. The acid was further identified by the preparation of benzhydryl *trans-trans*-muconate, m.p. and mixed m.p. 190° (the *cis-cis*-ester melts at 156° and the *cis-trans*- at 142°). These isolation experiments strongly suggest that benzene urine contains only *trans-trans*-muconic acid.

Experiments on the injection of the three isomers of muconic acid

The estimation of cis-cis- (*and/or* cis-trans-) *muconic acid in the presence of* trans-trans-*muconic acid.* The colour reaction developed above for the estimation of the muconic acids could not be applied as such when more than one isomer occurred in the test solution, because of the different amounts of colour produced by the *cis*-acids at 100 and 160°. A solution of this difficulty, however, was found in the observation of Grundmann & Trischmann (1936) (cf. Elvidge *et al.* 1950*b*) that the *cis-cis*- and *cis-trans*-acids may be quantitatively converted to the *trans-trans*-acid by irradiation with ultraviolet light in the presence of small amounts of I_2. The conditions of this transformation when the acids occurred in urine were therefore investigated. When these conditions were known the *cis*-acids could then be estimated in the presence of the *trans-trans*-acid by first estimating the *cis*-acids in a mixture by the colour reaction with phenol at 100°, then irradiating with ultraviolet light to convert the *cis*-acids into the *trans-trans* and finally estimating the total muconic acid as the *trans-trans*-isomer by the colour reaction with phenol carried out at 160°.

In pure solution it was found that 100% conversion of the *cis*-acids (5 ml. of 1 mg./ml. solution) to the *trans-trans*-acid occurred on irradiation from above, for 1–2 hr., of the solution containing 0·5 ml. of 0·1% ethanolic I_2 in small beakers placed 10 cm. below the ultraviolet lamp. In all experiments a Gallenkamp ultraviolet lamp was used, stated by the makers to contain a '125 watt mercury discharge unit enclosed by an ultraviolet glass filter from which there is practically no visible light, 95% of the radiation being at 3650 A.'. The solutions were thus in effect irradiated by light of wavelength 365 mμ. which is some distance from the absorption maximum of muconic acid which is at 260 mμ. However, irradiation of the solutions with a carbon arc lamp produced no better results than the Gallenkamp lamp.

It was also found that the isomerization could be carried out just as efficiently in 3 × $\frac{5}{8}$ in. test tubes kept upright and irradiated from above, as in open beakers. Furthermore, the isomerization took place equally well in dilute urine, dilute H_2SO_4 or $NaHCO_3$ and in the presence of $HgCl_2$ which was used as an antiseptic for the urines. The amount of I_2 used as a catalyst, however, was important. In the first place it was found that solutions of I_2 in KI interfered with the subsequent development of the red colour with phenol, but ethanolic solutions of I_2 were satisfactory because the I_2 could be removed by evaporation at the end of the irradiation period.

The effect of I_2 was investigated as follows. *cis-trans*-Muconic acid was dissolved in tenfold diluted normal rabbit urine to a concentration of 0·1 mg./ml. To 1 ml. of this solution there was added from 0·05 to 0·2 ml. of 1% ethanolic I_2 solution. These solutions were irradiated with ultraviolet light for 2 hr. in 3 × $\frac{5}{8}$ in. test tubes, held upright 10 cm. below the lamp as mentioned above. The solutions were then dried *in vacuo* at room temperature over P_2O_5 and the residue condensed with 0·5 g. phenol and 0·1 ml. conc. H_2SO_4 at 160° for 20 min. as described for the estimation of *trans-trans*-muconic acid. The red colour was measured as before. Similar solutions without the irradiation were dried *in vacuo* and condensed with phenol at 100° as in the estimation of *cis-cis*-muconic acid. The results are shown graphically in Fig. 6, which shows that in 1 ml. of diluted rabbit

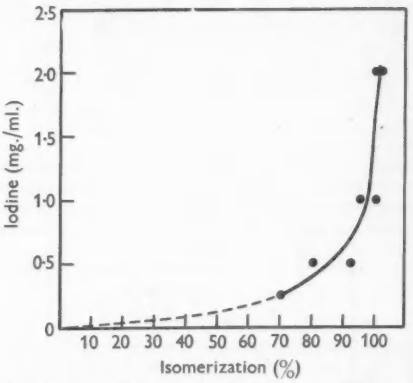


Fig. 6. The effect of the amount of iodine present on the isomerization by ultraviolet light of *cis-cis*- to *trans-trans*-muconic acid (0·1 mg./ml.).

urine 1 mg. of I_2 (0·1 ml. of 1% ethanolic I_2) gives 100% conversion of 0·1 mg. of the *cis*-acids to the *trans-trans*-acid on irradiation for 2 hr. This somewhat large amount of I_2 is necessary to allow for the consumption of I_2 by normal rabbit urine (approx. 4·5 ml. of 1% ethanolic I_2/100 ml.) and for that lost by evaporation during the irradiation.

Mixtures of all three acids were now estimated in tenfold diluted rabbit urine. The procedure which was finally used for benzene urine was to take 1 ml. of urine appropriately diluted with water so as to contain 0·1 mg. of muconic acid and the procedure for the estimation of *cis-cis*-muconic acid was then followed. To another 1 ml. of the urine made up in the same way there was added 0·1 ml. of 1% ethanolic I_2, then irradiated for 2 hr. with ultraviolet light and the procedures for the estimation of *cis-cis*- and *trans-trans*-muconic acids followed. With still another 1 ml. the *trans-trans*-estimation was carried out without irradiation. A blank estimation was also carried out on 1 ml. urine containing 0·1 ml. 1% I_2 but without muconic acid, condensations being carried out at 100 and 160°. With benzene urine the blank estimations were carried out on urine collected prior to dosing. Recovery experiments are given in Table 3.

Injection of trans-trans-*muconic acid.* The acid was dissolved in an equivalent amount of saturated aqueous $NaHCO_3$. Such a solution contained approx. 100 mg./ml. This solution was injected intraperitoneally into rabbits, each rabbit receiving 100 mg. muconic acid/kg. The urines were collected daily in flasks containing 0·5 ml. of 1%

Table 3. *Recovery of isomeric muconic acids from diluted rabbit urine*

(*E* values are for the red colour obtained by condensation of muconic acid with phenol and H_2SO_4 and then dissolving in ethanol (see text). Corrected values (for 160° condensations) are obtained by subtracting the urine blank values.)

Material	Ultraviolet irradiation treatment	*E* at 520 mμ. Condensation temperature: 100° for 6 hr.	160° for 20 min.: Found	160° for 20 min.: Corrected	Recovery (%)
In aqueous solution (1 ml.):					
0·1 mg. *cis-cis*	None	0·275	0·53	—	—
0·1 mg. *cis-trans*	None	0·28	0·54	—	—
0·1 mg. *trans-trans*	None	0·02	0·27	0·27	—
In urine (1 ml.) + I_2 (0·1 ml.):					
None (urine blank)	None	0·155	0·185	—	—
0·1 mg. *cis-cis*	2 hr.	0·185	0·445	0·26	95
0·1 mg. *cis-trans*	2 hr.	0·160	0·43	0·245	88
0·05 mg. each of *cis-cis* and *trans-trans*	2 hr.	0·135	0·485	0·300	109
0·05 mg. each of *cis-trans* and *trans-trans*	2 hr.	0·140	0·435	0·250	91

Table 4. *Excretion of muconic acid by rabbits after the intraperitoneal injection of the three isomeric muconic acids*

Muconic acid injected	Rabbit no.	Weight (kg.)	Dose (mg.)	Muconic acid recovered: As *cis-cis* or *cis-trans* (mg.)	As *trans-trans* (mg.)	Percentage of dose	
trans-trans	75	2·8	250	0	137	56	52
	82	2·8	250	0	119	48	
	87	2·2	175	0	86	49	
	67	2·45	240	0	132	55	
cis-cis	75	2·8	250	154	0	62	66
	82	2·8	250	179	0	72	
	86	3·1	280	189	0	66	
	51	2·4	240	145	0	61	
cis-trans	75	2·8	250	150	0	60	55
	82	2·8	250	118	0	47	
	86	3·1	280	159	0	58	

Table 5. *Estimation of* cis-cis-*muconic acid in the urine of rabbits injected with the* cis-cis-*acid, and demonstrating the absence of the* trans-trans-*acid*

(Each rabbit was injected with 100 mg./kg. of *cis-cis*-muconic acid. *E* values are for the red colour obtained by condensation of muconic acid with phenol and H_2SO_4 and then dissolving in ethanol.)

Rabbit no.	Ultraviolet irradiation treatment of urine	*E* at 520 mμ. condensation temperature: 100° for 6 hr.	160° for 15 min.
75	None	0·415	—
	2 hr.	—	0·45
82	None	0·48	—
	2 hr.	—	0·48
86	None	0·51	—
	2 hr.	—	0·50

$HgCl_2$. Each urine was then diluted to 1000 ml. with water, and 1 ml. of this diluted solution used for the estimation of muconic acid. Only traces of colour which were within the error of the method were obtained when the condensation with phenol was carried out for 6 hr. at 100°, thus showing the absence of *cis-cis-* and *cis-trans*-muconic acid. The main colour production occurred on condensing with phenol at 160° for 20 min. In four experiments an average of 52% of the injected *trans-trans*-muconic acid was recovered in the urine as such within 24 hr. of dosing, there being no conversion into the other isomers (see Table 4). On continuous ether extraction of the urine after acidification, only the *trans-trans*-isomer was isolated, m.p. and mixed m.p. 300°; 80% of the original urine yielded 204 mg. of the acid which is equivalent to 36% of that injected.

Injection of cis-cis-*muconic acid.* In a similar manner, 100 mg./kg. of *cis-cis*-muconic acid was injected into rabbits and the urine collected and diluted as before. On developing the colour with phenol at 100° for 6 hr. an average of 66% of the injected acid was recovered unchanged (see Table 4). On developing the colour at 160° for 20 min. an increase in the amount of colour was observed, but after irradiation of the urine with ultraviolet light and then estimating *trans-trans*-muconic acid, the colour produced was the same as for the procedure for the *cis-cis*-acid (see Table 5). This indicated that urine contained no *trans-trans*-acid.

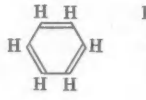
On continuous extraction of the urine with ether, the muconic acid isolated had m.p. 189° after two recrystalliza-

tions from ethanol. It was thus not *trans-trans*, but one or a mixture of the *cis*-acids.

Injection of cis-trans-*muconic acid.* In a similar way, *cis-trans*-muconic acid was injected and 55% was found again in the urine (Table 4). Ether extraction of the urine yielded the *cis-trans*-acid, m.p. 189–190°, after two recrystallizations from water (yield approx. 50% of dose).

DISCUSSION

The discovery by Elvidge *et al.* (1950*b*) that mere recrystallization of *cis-cis*-muconic acid (II and II*a*) from water yields the *cis-trans*-isomer (III) makes much of the earlier biochemical work, in which the so-called *cis-cis*-muconic acid was used, difficult to interpret. If samples of *cis-cis*-muconic acid which had been crystallized from water were used, it appears very probable that earlier workers were handling either mixtures of the *cis-cis*- and *cis-trans*-isomers or the *cis-trans*-acid alone. This point is an important one since Evans & Smith (1951) have shown that the *cis-cis*-acid behaves biologically in a different manner from the *cis-trans*-acid.



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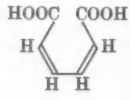

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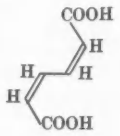
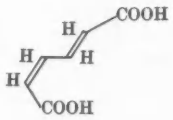
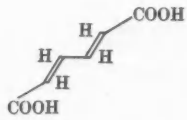


II*a*



III



IV

(Elvidge *et al.* (1950*b*) suggest that the *cis-cis*- and *cis-trans*-acids probably occur in extended *s-trans*-configuration (II*a* and III). II is the *s-cis*-configuration of *cis-cis*-muconic acid showing its relationship to benzene, I.)

The present work confirms the findings of earlier workers that only *trans-trans*-muconic acid (IV) is found in the urine of rabbits receiving benzene (I). Furthermore, evidence has been presented to show that this isomer could not have arisen by isomerization in the urine of the *cis-cis*-isomer after excretion. It is also shown that if any *cis-cis*-muconic acid were excreted it could not have been destroyed, before estimations could be carried out, by micro-organisms infecting the urine. *trans-trans*-Muconic acid, therefore, appears to be a genuine metabolite of benzene and not an artifact. By our method of estimation the amount of benzene, at a dose level of 0·5 g./kg., excreted as this acid, is about 0·5% (range 0·15–1·0%) of the dose. It is thus a minor metabolite of benzene.

Recently Ravdin & Crandall (1951) have obtained a water-soluble enzyme system from rat liver which oxidatively cleaves homogentisic acid to 4-fumarylacetoacetate,

$$COOHCH{:}CHCOCH_2COCH_2COOH.$$

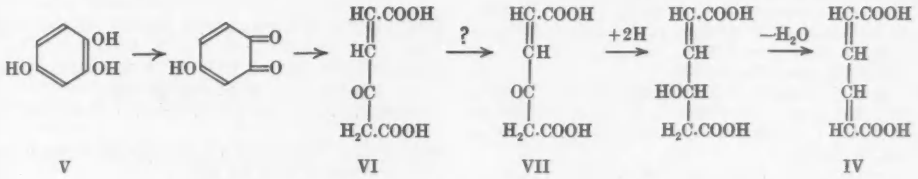
The configuration of the carbon-carbon double bond in this compound is *trans*, whereas the original configuration of this bond in the benzene ring of homogentisic acid is *cis*. Thus we have here another problem similar to that of the muconic acids.

Drummond & Finar (1938) concluded from their experiments that *cis-cis*-muconic acid was not isomerized to the *trans-trans*-acid in the animal organism. On injection of '*cis-cis*-muconic acid' they recovered by isolation in two experiments 47 and 45% of the acid unchanged. With injected *trans-trans*-muconic acid they recovered by isolation an average of 56% of doses of 800 mg., and similar results were obtained by Mori (1918) who recovered 55–60%. In most of these experiments the yields were based on crude materials and are therefore probably high. Our experiments on the injection of the three isomers in rabbits confirm the views of Drummond & Finar. Whichever isomer is injected, the same isomer is excreted (when allowance is made for the easy isomerization in aqueous solution of the *cis-cis*- into the *cis-trans*-acid). We found that at a dose level of 100 mg./kg., 50–65% of the acids were excreted unchanged in about 24 hr. The metabolites of muconic acid are not known, but it seems likely that the acid undergoes β-oxidation in the usual way, for Hensel & Reisser (1913) observed that in the perfused liver, muconic acid (presumably the *trans-trans*-acid as this was the only isomer known in 1913) gives rise to acetone. According to Raizon & Yamamoto (1940) perfusion of dog liver with the *trans-trans*-acid leads to the production of acetone bodies, but the *cis-cis*-acid does not. Mori (1918), however, reports that the *trans-trans*-acid does not increase the output of oxalic acid in rabbits.

These results lead to the conclusion that *cis-cis*-muconic acid is not necessarily an intermediate in the metabolism of benzene in the animal body. Evans & Smith (1951), however, have suggested from simultaneous adaptation studies that *cis-cis*-muconic acid is an intermediate in the microbiological oxidation of phenol, catechol and benzoic acid to β-ketoadipic acid. Hayaishi & Hashimoto (1950) have isolated *cis-cis*-muconic acid (or more probably *cis-trans*- in view of the treatment the material received during the isolation) from the oxidation of catechol by an enzyme from *Pseudomonas* sp. Thus *cis-cis*-muconic acid may be an intermediate in the bacterial oxidation of phenol,

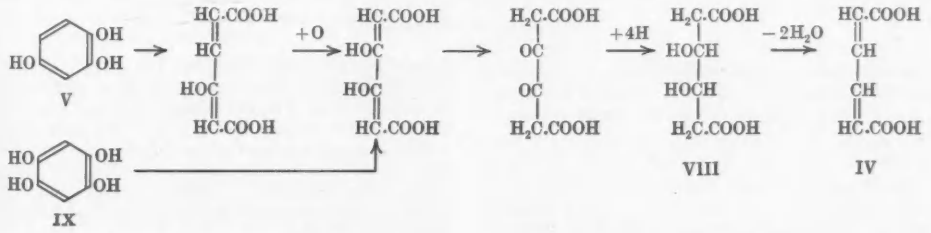
although its microbiological production from benzene itself has not yet been studied.

Pathways of metabolism of benzene to *trans-trans*-muconic acid without the intermediate formation of the *cis-cis*-acid can readily be postulated. Thus hydroxyquinol (V) which is a metabolite of benzene (Porteous & Williams, 1949*b*) could give rise to fumarylacetic acid (VII) in the same way as homogentisic acid gives rise to fumarylacetoacetic acid (Ravdin & Crandall, 1951), although one would expect maleoylacetic acid (VI) as an intermediate. Reduction followed by dehydration of fumarylacetic acid could then give rise to *trans-trans*-muconic acid (IV) thus:



V VI VII IV

There are obvious difficulties in this postulate, such as the conversion of maleoylacetic to fumarylacetic acid. Another possibility is the formation of $\beta\beta'$-dihydroxyadipic acid (VIII) which might give the *trans-trans*-acid on dehydration. Dihydroxyadipic acid could arise from hydroxyquinol or 1:2:4:5-tetrahydroxybenzene (IX) (this last phenol has not been found as a metabolite of benzene), thus:



According to Stanier, Sleeper, Tsuchida & Macdonald (1950) hydroxyquinol is probably not an intermediate in the bacterial oxidation of phenol and other compounds to β-ketoadipic acid. The latter is a common intermediate in the bacterial oxidation of several aromatic compounds, but we have never detected it in the urine of rabbits receiving benzene.

It is possible that the solution of the stereochemical problems involved in the formation of *trans-trans*-muconic acid from benzene in the intact animal and of fumarylacetoacetic acid from homogentisic acid in tissue extracts may lie in the chemistry of unsaturated hydroxy compounds which undergo ready isomerization.

SUMMARY

1. A colorimetric method for the determination of the three geometrical isomers of muconic acid has been studied. The method is based on the production of a red colour by heating muconic acid with phenol and concentrated sulphuric acid and then dissolving the product in absolute ethanol.

2. The same amount of colour is produced on heating the *cis-cis*- and *cis-trans*-acids at 100° for 6 hr. as on heating the *trans-trans*-acid at 160° for 20 min. By this method the *cis*-acids can be distinguished from the *trans-trans*-acid and estimated when they occur separately. This method has also been applied to urine.

3. When the *cis*-acids and the *trans-trans*-acid occur together the same colorimetric method can be used for their estimation, if the *cis*-acids are first estimated in the mixture and then converted to the *trans-trans*-acid by ultraviolet light followed by an estimation of the total muconic acid as the *trans-trans*-isomer.

4. By this colorimetric method it has been shown that rabbits receiving an oral dose of benzene excrete about 0·5% of the dose as *trans-trans*-muconic acid, there being none of the *cis*-isomers in the urine. *trans-trans*-Muconic acid was isolated from the urine and characterized as its benzhydryl ester.

5. It has been shown that if *cis-cis*-muconic acid had been excreted after administration of benzene it could not have been destroyed by bacteria.

6. The three isomers of muconic acid have also been injected into rabbits and no evidence was found for their *in vivo* isomerization, 50–65% of unchanged acid being recovered in the urine.

7. It is concluded that *trans-trans*-muconic acid is probably a true metabolite of benzene and is not produced *in vivo* from the *cis-cis*-acid. It is suggested that the *cis-cis*-acid need not be an intermediate in the formation of the *trans-trans*-acid in the animal body.

The expense of this work was in part defrayed by a grant from the Medical Research Council. We are grateful to Prof. R. Cruickshank and Dr R. Mendex for classifying the organisms found in stale rabbit urine, to Prof. W. C. Evans for the microbiological testing, of the urinary muconic acid and to Prof. R. P. Linstead, F.R.S., for access to information on the muconic acids prior to publication.

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The Separation of Esters of Choline by Filter-paper Chromatography

By V. P. WHITTAKER* and S. WIJESUNDERA
Department of Biochemistry, University of Oxford

(*Received* 18 *August* 1951)

The technique of paper chromatography (Consden, Gordon & Martin, 1944) has been applied in recent years to the separation of numerous classes of substances. It has now been applied to esters of choline in connexion with work on the identification of choline esters in tissue extracts and on the mechanism of enzymic reactions involving choline esters. Preliminary accounts have been given by Whittaker (1951) and Whittaker & Wijesundera (1951).

METHODS

Chromatographic procedure

Whatman no. 4 filter paper was used throughout. For preliminary screening of solvents, tall inverted beakers or wide-mouthed bottles with screw caps were employed as containers. Solutions of esters in water (10–50 μg. in 10 μl.) were delivered, 3 cm. from the lower edge, on to a rectangle of filter paper of suitable dimensions by means of an 'Agla' micropipette (Burroughs Wellcome Ltd.). The paper was rolled into a cylinder whose axis was parallel to the intended direction of solvent flow, and the apposed ends of the paper secured by wire staples. The cylinders were then placed upright in a pool of solvent at the bottom of the container and irrigated by capillary ascent, a 10 cm. run taking 2–4 hr. Promising solvents were then tried with longer runs, using both upward and downward irrigation in containers of conventional design.

Reading of chromatograms

Esters were rendered visible on the paper by three methods, one specific for carboxylic esters and certain other compounds containing an acyl linkage (e.g. lactones, acyl phosphates), the other two relatively non-specific.

Carboxylic reagent

The first method employed a reaction first used by Feigl, Anger & Frehden (1934, cf. Feigl, 1939) as the basis of a spot test, and later applied to the quantitative estimation of acyl phosphates (Lipmann, 1946) and acetylcholine (Hestrin, 1949). The reaction involves the conversion of the carboxylic ester into a hydroxamic acid with alkaline hydroxylamine, followed by the formation of a purple

* Now at Department of Physiology, Cincinnati University College of Medicine, Cincinnati 19, Ohio, U.S.A.

complex between the hydroxamic acid and Fe^{+++} in acid solution:

$$R.CO.OR' + NH_2OH \rightarrow R.CO.NHOH + R'OH$$

$$R.CO.NHOH + \tfrac{1}{3}Fe^{+++} \rightarrow R{-}C(=O)(-O)\,(Fe)\ (-NH{-}O)$$

In preliminary studies (by V.P.W.) the reagents of Hestrin (1949) were used. The papers were hung in a box inside a fume cupboard, and sprayed first with alkaline hydroxylamine, then, after 1 min., with acid $FeCl_3$. An initial brown colour (due to $Fe(OH)_3$) was replaced by yellow when sufficient acid had been delivered, the esters showing as purple spots. This method suffers from the following disadvantages: the spots are fugitive and must be outlined at once in pencil; the esters are soluble in the aqueous reagents and tend to diffuse away from their original positions; the reagents, being strongly alkaline and acid, are unpleasant to use. These disadvantages have been largely overcome, and the sensitivity considerably increased, by the use of non-aqueous reagents, which are also more stable.

Stock reagents. The following stock solutions are employed:

(*A*) Aqueous ethanolic hydroxylamine hydrochloride. Hydroxylamine hydrochloride (20 g.) is dissolved in water (50 ml.) and diluted to 200 ml. with 95% (v/v) ethanol. Stable when stored in the cold.

(*B*) Ethanolic KOH. The hydroxide (50 g.) is dissolved in the least quantity of water and diluted to 500 ml. with 95% (v/v) ethanol.

(*C*) Ethereal acid $FeCl_3$. Well powdered $FeCl_3.6H_2O$ (10 g.) is dissolved in 20 ml. 10N-HCl and shaken with 300 ml. ether until a homogeneous solution results. Stable indefinitely in well stoppered bottles.

Spray reagents. These were made from the stock solutions as follows:

(I) Ethanolic alkaline hydroxylamine. Stock solution *A* is mixed with twice its volume of solution *B* and filtered from precipitated KCl. Stable for over 2 weeks in the cold.

(II) Stock solution *C* used directly.

Procedure. The dry chromatograms were clamped in a box, reagent I applied thinly and evenly with an atomizer supplied with compressed N_2, dried briefly and sprayed with reagent II. The esters showed up almost at once as purplish spots on a yellow ground.

Sensitivity. As little as 0·5 μg. acetylcholine/cm.^{2} gave a faint fugitive spot and 2 μg./cm.^{2} gave distinct spots which remained visible for several weeks. With weaker reagents, larger volumes have to be delivered on to the papers, thus making them wet and increasing the risk of diffusion. Ethereal ethanolic hydroxylamine in place of reagent *A* gave fugitive spots.

Iodine method

Brante (1949) found that nitrogenous bases could be separated by paper chromatography and visualized as brown spots by immersing the paper in I_2 vapour. As pointed out by Marini-Bettolo-Marconi & Guarino (1950), this method is non-specific and can be applied to a wide variety of compounds. On exposing the treated paper to the air, the colour fades and the paper may then be treated with another reagent or assayed pharmacologically. We have found the iodine method particularly useful in conjunction with other methods, and exact coincidence was obtained in areas developed by the iodine and carboxylic methods.

Phosphotungstic acid-stannous chloride method

The method of Chargaff, Levine & Green (1948) for choline was found to be applicable to choline esters. It was found to be less convenient for our purposes than the other two methods, being less specific and less sensitive; moreover the washing-out stages were attended with a risk of damage to the chromatograms.

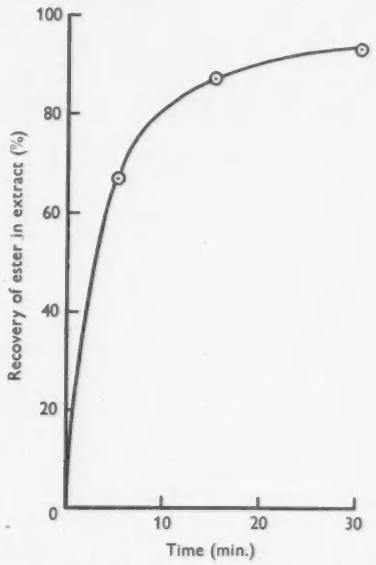

Fig. 1. Time required for extraction of acetylcholine from filter paper.

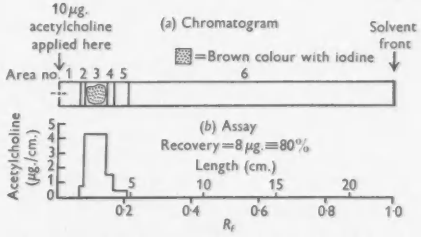

Fig. 2. (*a*) Chromatogram of acetylcholine. Solvent, *n*-butanol saturated with water (ascending). (*b*) Distribution of acetylcholine on chromatogram, as revealed by pharmacological assay (guinea pig ileum).

Pharmacological assay

Esters which are pharmacologically active can be localized by bioassay. Fig. 1 shows that elution of acetylcholine from filter paper with water is 93% complete in 30 min. In this experiment a series of spots, each containing 150 μg. acetylcholine chloride placed on filter paper, were run in *n*-butanol saturated with water. The papers were dried and the positions of the spots located by developing

control spots with iodine. Areas corresponding to the spots were then cut up and extracted in 3 ml. water each for various lengths of time. The acetylcholine content of samples withdrawn at 5, 15 and 30 min. was determined by the method of Hestrin (1949). Fig. 2 (*a*) is the tracing of a chromatogram of 10 μg. acetylcholine run in *n*-butanol saturated with water. The chromatogram was cut into numbered areas; each area was cut up and eluted for 30 min. in 2 ml. of a saline solution acidified to pH 4, the pH at which acetylcholine has maximum stability. Each extract was assayed in the usual manner using a guinea pig ileum preparation. The result of the assay is shown in the lower half of the figure (Fig. 2*b*). It will be seen that good localization and recovery of the acetylcholine are obtained.

RESULTS

The R_F values of a number of esters in different solvents are presented in Table 1. Essentially the same values were obtained for an ester whether run alone or as a component in a mixture, but the values obtained in any one experiment were found to be influenced by the usual factors and rigid control of temperature, length of run and composition of solvent were necessary to secure uniform results.

0·13–0·16 above those of the chlorides. Increasing water content raised R_F values and reduced separation; with ethanol and acetone it also reduced the 'tailing' which occurred with the pure solvents. Pyridine-, collidine-, lutidine-, phenol-, benzyl alcohol-, furfuryl alcohol- or dioxan-water mixtures proved either unsatisfactory or inferior in resolving power to those included in the table. Solvents containing ammonia destroyed choline esters, which are markedly alkali-labile.

DISCUSSION

The results presented above show that it is possible to separate esters of choline using paper chromatography, the best solvents being the less freely water-soluble alcohols, either alone or in combination. Three methods of chemical identification have been described; one, using alkaline hydroxylamine and acid ferric chloride, is specific for compounds containing the acyl linkage and should be applicable to other esters of low volatility, and possibly also to acyl phosphates and lactones, which can be estimated with the reagents (Kent, 1951).

Table 1. *Mean R_F values of choline esters and related compounds*

(Length of run: 25 cm. (upward); 35 cm. (downward); (*a*) denotes upward; (*d*) downward irrigation. Composition of solvents given as proportions by volume of constituents. Whatman no. 4 filter paper.)

Compound	*n*-Butanol saturated with water		*n*-Butanol-*n*-propanol-water (4:2:1)	*n*-Propanol-water (9:1)	*n*-Propanol-formic acid-water (8:1:1)	*n*-Propanol-benzyl alcohol-water (5:2:2)	
	(*d*)	(*a*)	(*a*)	(*a*)	(*a*)	(*d*)	(*a*)
Chlorides							
Acetylcholine	0·09	0·14	0·17	0·24	0·46	—	0·33
Acetyl-β-methylcholine	0·15	0·19	0·23	0·35	0·56	—	0·37
Propionylcholine	0·17	0·22	0·27	0·35	0·59	—	—
Butyrylcholine	0·22	0·28	0·29	0·43	0·66	—	0·46
Benzoylcholine	0·23	0·28	0·30	0·43	0·65	—	0·49
Lactylcholine	—	0·18	—	—	—	—	—
Valerylcholine	—	—	0·55	—	—	—	—
Choline	0·06	0·09	0·13	0·24	0·38	—	0·25
Perchlorates							
Acetylcholine	0·22	0·30	0·34	0·40	0·59	—	0·48
Acetyl-β-methylcholine	0·31	—	0·38	0·52	—	—	—
Propionylcholine	0·32	0·42	0·39	0·49	0·67	—	0·54
Butyryl-β-methylcholine	0·49	—	0·59	0·70	0·75	—	—
Lactylcholine	—	0·34	—	—	—	—	—
Succinylmonocholine	Unsatisfactory		0·04	Unsatisfactory	0·62	0·10	0·17
Succinyldicholine	Unsatisfactory		0·12	Unsatisfactory	0·30	0·19	0·30
Choline	0·21	0·24	0·31	0·39	0·60	0·50	0·46
β-Methylcholine	0·29	0·45	0·40	0·48	0·56	—	—

Increasing molecular complexity leads to increased R_F values. In *n*-butanol, for example, each additional CH_2 (starting with acetylcholine) increases the R_F by approximately 0·07; isomers, e.g. propionylcholine and acetyl-β-methylcholine ($[Me_3N^+.CH_2.CHMe.OAc]OH^-$), having closely similar values. The associated anion influences the R_F; in *n*-butanol, the R_F's of perchlorates are

SUMMARY

1. Choline esters can be separated from each other and from choline by the technique of paper chromatography. *n*-Propanol- and *n*-butanol-water mixtures, with or without the addition of a third component such as benzyl alcohol or formic acid, gave the best separations.

2. The esters were detected on the paper by an adaptation of the hydroxylamine-ferric chloride test for carboxylic esters, by the iodine method of Brante (1949), by the phosphotungstic acid-stannous chloride method of Chargaff *et al.* (1948) and, in the case of acetylcholine, by pharmacological assay.

We are grateful to the Medical Research Council for a grant (V.P.W.) and the University of Ceylon for a travelling fellowship (S.W.).

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The Metabolism of ^{14}C-Labelled Bicarbonate in the Cat

By H. L. KORNBERG, R. E. DAVIES and D. R. WOOD
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, and Department of Pharmacology and Therapeutics, The University, Sheffield 10

(*Received* 12 *September* 1951)

Studies on the metabolism of ^{14}C-labelled urea in mice (Leifer, Roth & Hempelmann, 1948; Skipper, Bennett *et al.* 1951) and cats (Kornberg, Davies & Wood, 1951*b*) have shown conclusively that urea is broken down in the mammalian body. Its rate of breakdown in these experiments has been measured from the rate of expiration of the ^{14}C-labelled carbon dioxide produced. In order to interpret the results of such studies on cats, it was necessary to have information on the rate of excretion from the blood of ^{14}C-labelled carbon dioxide, and on the rate of incorporation of ^{14}C into urea. The results of earlier studies on mice and rats injected with ^{14}C-labelled bicarbonate (Armstrong & Schubert, 1949; Armstrong & Zbarsky, 1949; Gould, Sinex, Rosenberg, Solomon & Hastings, 1949; Greenberg & Winnick, 1949; Schubert & Armstrong, 1949; Skipper, White & Bryan, 1949; Skipper, Nolan & Simpson, 1951; Skipper, Bennett *et al.* 1951) did not supply the required data, because of the great differences in body size between these animals and cats, and because the isotope in these experiments had been administered intraperitoneally.

Experiments are described in which ^{14}C-labelled sodium bicarbonate was injected intravenously into anaesthetized cats and the expired carbon dioxide continuously collected in a special respiration circuit. By this technique, information was obtained on the rate of expiration of labelled carbon dioxide from ^{14}C-labelled bicarbonate, and on the existence of exchange mechanisms between blood and tissue carbon dioxide in the animal. The results of these studies are very similar to those obtained independently in human subjects (Hellman, 1951). From determinations of blood urea and bicarbonate it was also possible to measure the rate of incorporation of ^{14}C into urea synthesized during the experiment, and to confirm that the urea carbon is derived from carbon dioxide.

Part of this work has been communicated to the Biochemical Society (Kornberg, Davies & Wood, 1951*a*).

EXPERIMENTAL

Treatment of cats. A weighed cat was anaesthetized with ether followed by chloralose (75 mg./kg. body weight) and was placed on an operating table covered by a large stainless steel tray to avoid contamination of the laboratory. Cannulae were inserted into the trachea for collection of expired CO_2, into the right external jugular vein for administration of intravenous injections, and into the right femoral artery for collection of blood samples during the course of the experiment. Both ureters were tied off. The animal was then connected through its tracheal cannula to the respiration circuit schematically represented in Fig. 1.

Respiration circuit. This system is so constructed that atmospheric pressure is maintained at the tracheal cannula, so that there is no resistance to free respiration. This is achieved by the two aquarium pumps P_1 blowing, and P_2 drawing air through the system at approximately 400 ml./min. Constancy of the pressure inside the circuit is controlled by three leaks L and a rubber balloon B which acts as a gas reservoir, these being placed at points which do not at any time come into contact with $^{14}CO_2$. Two soda-lime towers S remove atmospheric CO_2 from the incoming air, which can

be freely inspired by the cat through a valve inserted in the tracheal cannula. The expired CO_2 is absorbed in one or other of the two absorption trains A_1 or A_2, only one of which is in circuit at any one time. They are changed by means of the two three-way taps T_1 and T_2, and replaced by fresh absorption trains at frequent known intervals. The air, now again CO_2-free, is passed down a water pump to avoid the possibility of accidentally contaminating the laboratory.

evolved was quantitatively absorbed in a slight excess of 0·3N-NaOH, in the apparatus described below. The liquid was adjusted to pH 9·3 by careful addition of 0·05N-HCl, using thymolphthalein as internal indicator. This gave a solution which was sufficiently alkaline to retain its $^{14}CO_2$ content, but which could be safely injected into the cat. Samples (1 ml.) of solutions prepared as above, containing 0·3–0·6 mg. carbon, and corresponding to 510000, 700000

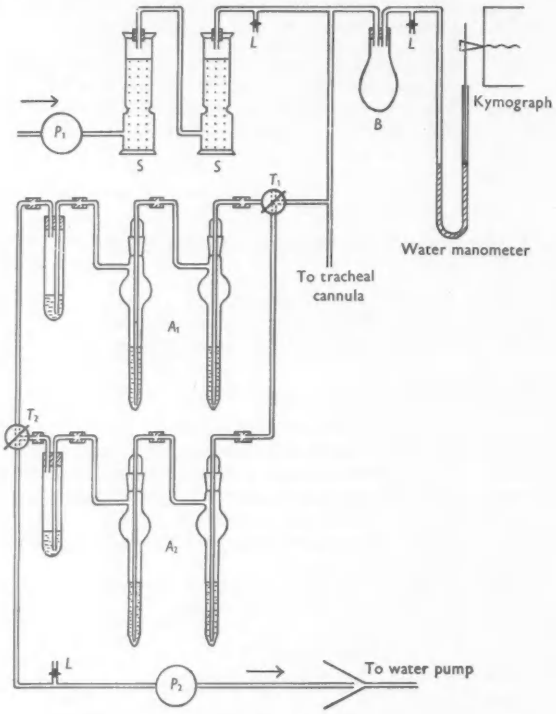

Fig. 1.

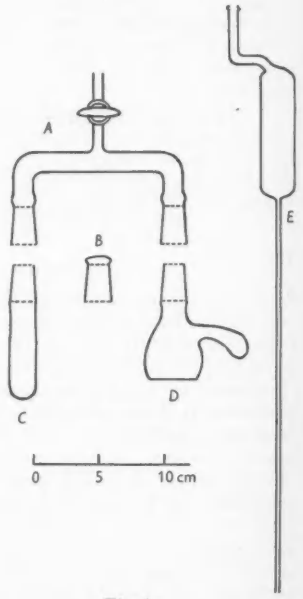

Fig. 2.

Fig. 1. Respiration circuit for absorption of expired $^{14}CO_2$. P_1, P_2, aquarium pumps; *L*, leaks with screw-clips; *B*, rubber balloon acting as gas reservoir; *S*, soda-lime towers; T_1, T_2, three-way taps; A_1, A_2, absorption trains (only A_2 is in circuit).

Fig. 2. *A*, vacuum transfer unit; *B*, cap for small tubes; *C*, small tube for $Ba(OH)_2$; *D*, side-bulb cup; *E*, long pipette fitted with rubber bulb, for removal of liquid from absorption units.

The absorption trains, A_1 and A_2, consist of two all-glass units which are connected in series, and each of which contains 10 ml. approx. 6N-NaOH and 3–4 drops 0·75% (w/v) egg albumin. This latter substance acts as a frothing agent and, by forming a layer of alkaline bubbles in the units, ensures complete absorption of the CO_2. The efficiency of absorption is checked by a third tube containing 5 ml. saturated $Ba(OH)_2$, which solution should, and in fact does, remain unclouded throughout the period of absorption.

Preparation and injection of labelled materials. $NaH^{14}CO_3$ solutions were prepared from $Ba^{14}CO_3$. Samples (7–15 mg.) of $Ba^{14}CO_3$ (equivalent to 50–100 μc.) were acidified with lactic acid (British Pharmacopoeia) *in vacuo*, and the $^{14}CO_2$ and 1020000 counts/min. respectively under our conditions of radio-assay (see later), were injected quantitatively in the three experiments on HCO_3^- metabolism to be described.

Collection and analysis of samples

Carbon dioxide. When the ^{14}C-labelled material had been injected, the expired CO_2 was continuously collected over the next 4–5 hr., any one absorption train being used for periods varying from 5 to 20 min. The $NaOH$-Na_2CO_3 solutions in the units were then quantitatively transferred by means of long pipettes with a rubber bulb at one end (Fig. 2*E*) to ampoules which were immediately sealed. The

units were then washed, fresh NaOH and frothing agent introduced, and the trains reassembled for use later in the experiment.

The liquid obtained from any one absorption train was made up to 50 ml. with water, and 1–2 ml. samples taken for analysis. All analyses were carried out in an apparatus modified by Davison from that of Barker (described by Kamen, 1948). These samples were pipetted into the main compartment of a side-bulb cup (Fig. 2*D*) and approximately 1 ml. lactic acid introduced into the side bulb by means of a bent Pasteur pipette. The side-bulb cup was attached to the transfer unit (Fig. 2*A*), a small tube (Fig. 2*C*) containing 3 ml. 0·22 N-$Ba(OH)_2$ and 2 drops thymol blue, being attached to the other end. The system was evacuated, the tap closed and the contents of the side bulb tipped into the main compartment. CO_2 was rapidly evolved and rapidly absorbed in the $Ba(OH)_2$. Quantitative absorption was ensured by warming the side-bulb cup and transfer unit in the hand, the system being shaken continuously for 5 min. whilst the small tube was cooled under running cold water. CO_2-free air was admitted into the system, and the small tube, which contained $BaCO_3$, was detached and stoppered with a flat cap (Fig. 2*B*). The amount of CO_2 absorbed was determined by titration of the unneutralized $Ba(OH)_2$ with 0·20 N-HCl. The precipitate was washed twice with water and once with acetone in a centrifuge, suspended in acetone, and plated on weighed aluminium disks, as described later.

At various times during the experiments, blood samples (1 ml.) were taken from the arterial cannula and received in stoppered tubes containing 0·2 ml. N-NaOH to prevent loss of CO_2. The tubes were stored at −12°. When required for analysis, the frozen clotted samples were thawed, homogenized with 1 ml. 1·7 N-NaOH and 4 ml. water, washed into measuring tubes with 1 ml. water and the volumes read. About half the liquid was measured into the main compartment of a double-armed Warburg vessel, with 1 ml. 3M-acetate buffer, pH 5·0, in one side arm and 0·8 ml. jack bean urease solution buffered to pH 5·0 (Davies & Kornberg, 1951) in the other. The vessels were equilibrated at 25° and first the buffer was added from one side arm. The amount of gas evolved gave the 'total CO_2' content of the sample. The 'total CO_2' content of any tissue was defined as the acid-volatile CO_2, which included dissolved CO_2, H_2CO_3, HCO_3^-, CO_3^{--} and carbamino compounds. As alkali had been added initially to all samples, the 'total CO_2' at the time of analysis was present in the form of CO_3^{--}.

When equilibrium had again been reached, the jack bean urease was added from the other side arm. The urea in the sample was hydrolysed, and an equivalent amount of CO_2 evolved. These estimations were accurate to ±5%.

Another sample of the dilute blood homogenate, together with 1 ml. 0·125M-Na_2CO_3 (containing 1·5 mg. C, added as carrier) was added to a side-bulb cup, and 1 ml. 1·5 N-H_2SO_4 was pipetted into the side bulb. The side-bulb cup was attached to one end of a transfer unit, a tube containing 3 ml. 0·22 N-$Ba(OH)_2$ and 2 drops thymol blue attached to the other, the system evacuated and the cup contents mixed. The CO_2 evolved was assayed by the techniques described. The cup was detached, 2 ml. 0·125M-Na_2CO_3 and 0·5 ml. 3M-acetate buffer, pH 5·0, were added to the contents and the system was evacuated. Any trace of radioactive C remaining in the system as bicarbonate had thus been diluted with 3 mg. non-radioactive C, which was then driven off on evacuation. CO_2-free air was admitted, 0·5 ml. 1·0% (w/v) urea solution was added to the contents of the cup, and 1 ml. jack bean urease solution pipetted into the side bulb. The cup was attached to one end of another transfer unit, a tube containing $Ba(OH)_2$ and thymol blue as above being attached to the other end. The system was evacuated and the cup contents mixed.

After 2 hr. all the urea in the sample had been decomposed, and the quantity and radioactivity of the $^{14}CO_2$ evolved was determined.

Tests showed that this procedure made it possible to determine independently highly active HCO_3^- and urea of low activity, present in the same sample of blood, without the results being vitiated by contamination.

Samples (1 g.) of cat tissues were taken during the experiments, blotted to remove adherent blood, and stored in weighed tubes containing 5 ml. 2 N-NaOH to retain all acid-volatile CO_2. The tissues disintegrated on standing and were washed quantitatively into the main compartments of side-bulb cups with addition of 1 ml. 0·125M-Na_2CO_3 to act as a carrier. The tissue 'total CO_2' was decomposed by addition *in vacuo* of lactic acid from the side bulb, and the $^{14}CO_2$ evolved was measured.

Measurement of radioactivity

The washed $BaCO_3$ samples were suspended in acetone, shaken vigorously in the small stoppered tubes, and poured into stainless-steel cylinders which fitted firmly into the circular grooves of weighed aluminium plates. Acetone leaked slowly past the junction of cylinder and plate, leaving the fine precipitates of $BaCO_3$ (20–30 mg.) deposited on the plates as uniform layers of area 3·8 sq.cm. The cylinders were covered and allowed to dry at room temperature overnight. When dry, the cylinders were removed, the plates weighed, and the radioactivity assayed with a thin mica-window Geiger-Müller counter, corrections having been made for background counts and for the dead-time of the system. At least 5000 counts were taken for each sample, giving a standard error of less than 1·5%. The efficiency of counting was frequently checked by counting a uranium standard. There was no detectable loss for at least 3 days in the activities of the samples, kept covered in Petri dishes, through exchange of their ^{14}C content with atmospheric CO_2.

All counts were corrected to infinite thickness for self-absorption in the $BaCO_3$ by means of an experimentally determined activity-saturation curve (cf. Calvin, Heidelberger, Reid, Jolbert & Yankwich, 1949), and the specific activities expressed as the corrected counts/min./mg. C. All analyses and counts were performed in duplicate and agreed to ±3%.

RESULTS

Expiration of $^{14}CO_2$

After intravenous injection of ^{14}C-labelled sodium bicarbonate, the rate of expiration of $^{14}CO_2$ is initially very rapid, and corresponds to an exponential equation with three components. The curves obtained in our experiments were found empirically to correspond within 2% to the function

$$(100 - P) = 28{\cdot}4e^{-0{\cdot}243t} + 41{\cdot}5e^{-0{\cdot}0234t} + 30{\cdot}2e^{-0{\cdot}00119t},$$

where P is the percentage of injected ^{14}C left in the cat at t min. after the injection (Fig. 3). The three components therefore have half-lives of 2·85, 29·6 and 582 min. respectively. In the first 30 min., 50 % of the injected ^{14}C is expired, the total output over the 5 hr. experiments being 79–81 %.

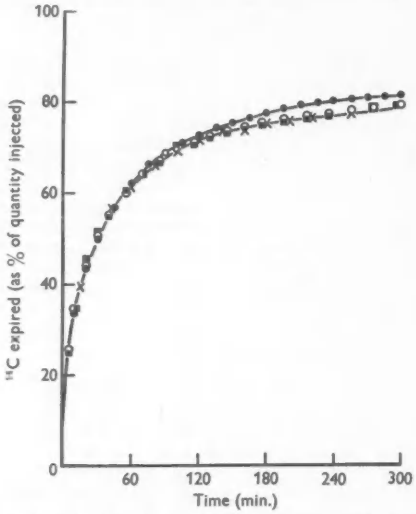

Fig. 3. Expiration of $^{14}CO_2$ after intravenous injection of $NaH^{14}CO_3$. ○—○, cat *A*, 1·83 kg.; ●—●, cat *B*, 1·55 kg.; ×—×, cat *C*, 1·69 kg.; ■—■, curve of $(100\text{-}P) = 28{\cdot}4e^{-0{\cdot}243t} + 41{\cdot}5e^{-0{\cdot}0234t} + 30{\cdot}2e^{-0{\cdot}00119t}$, where P is the percentage of injected ^{14}C remaining in cat after t min.

Semi-logarithmic plots of the specific activities of the expired $^{14}CO_2$ against time (Fig. 4) give smooth curves. Analysis of the radioactivity of the blood 'total CO_2' showed that the specific activities of expired CO_2 and blood 'total CO_2' are identical at any time, and these curves thus also represent the change in the specific activities of blood 'total CO_2' with time. The three curves obtained in our experiments cut the ordinate at specific activities of approx. 16 000, 16 000 and 28 000 counts/min./mg. carbon respectively. As the blood volume of the cat is approx. 70 ml./kg. body weight, and as the amounts of bicarbonate injected were negligible, this corresponds in each case to a blood 'total CO_2' concentration of 24–27 mM. This is the 'total CO_2' content of blood (Krebs, 1950) and shows that only at zero time is the radioactive material present entirely in the blood. After 3 hr. the rate of expiration of $^{14}CO_2$ is nearly constant and is less than 2 % of the injected isotope/hr. The blood and various soft tissues, which were weighed, have low, slowly decreasing, activities in their 'total CO_2' at this time (Table 1), and after 4 hr. together contain approx. 1 % of the injected ^{14}C, i.e. approx. 5 % of the ^{14}C still remaining in the cat. The activity of bone is 15–40 times that of any other tissue, and, since bone

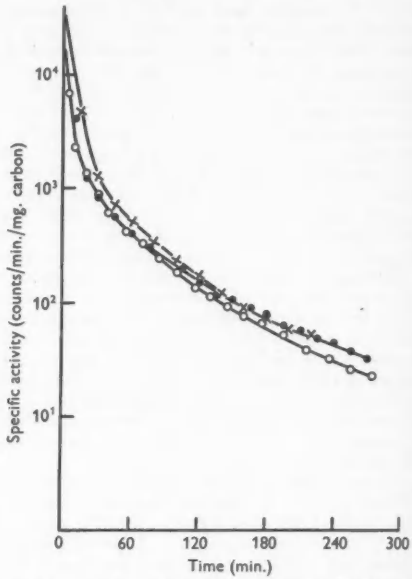

Fig. 4. Changes in specific activity of expired $^{14}CO_2$ after intravenous injection of $NaH^{14}CO_3$. ○—○, cat *A*, 1·96 kg.; ●—●, cat *B*; 1·55 kg.; ×—×, cat *C*, 1·69 kg.

forms approx. 18 % of the body weight of the animal (Custor, 1873) over 6 % of the injected ^{14}C, i.e. over 30 % of the isotope still remaining in the cat, is present in this tissue at this time.

Table 1. *Radioactivity of 'total CO_2' of tissues after intravenous injection of $NaH^{14}CO_3$ into a cat*

(Cat, 1·5 kg., received 1×10^6 counts/min. intravenously at time 0.)

Time (min.)	Tissue radioactivity (counts/g. wet wt.)				
	Blood	Jejunum	Muscle	Marrow	Femur
32	360	143	—	—	—
58	155	84	85	—	—
91	82	43	—	—	—
120	51	28	23	—	—
150	30	15	—	—	—
182	21	11	10	—	—
240	13	6	5	11	220

Urea synthesis

As the ureters of the experimental animals were tied at the start of each experiment, no ^{14}C was lost in the urine, and urea synthesis could be followed

both by the rise in the concentration and radioactivity of blood urea (Table 2). The urea synthesized in the early stages of the experiments is formed from CO_2 which is in equilibrium with blood bicarbonate of high specific activity, and the major part of the incorporation of ^{14}C into urea occurs in the first 40 min., the blood urea by this time containing 2·0 % of the ^{14}C injected. After 5 hr. 3·1 % of the administered isotope was incorporated into urea.

Table 2. *Concentration and specific activity of blood urea after intravenous injection of* $Na^{14}HCO_3$

(Cat I (1·55 kg.) received $5{\cdot}1 \times 10^6$ counts/min., cat II (1·83 kg.) $7{\cdot}0 \times 10^5$ counts/min., at time 0. Ureters tied.)

Cat no.	Time (min.)	Blood-urea concentration (mg./100 ml.)	Specific activity (counts/min./ mg. carbon)	Percentage of injected ^{14}C incorporated in urea
I	0	31·0	0·00	0·00
	5	31·0	56·8	0·80
	16	32·3	102	1·50
	25	32·6	117	1·74
	40	35·5	124	2·00
	70	36·0	145	2·37
	100	36·5	154	2·56
	130	42·0	150	2·67
	160	40·0	154	2·80
	192	44·0	143	2·87
	220	47·5	135	2·91
	250	47·7	136	2·96
	280	50·1	134	3·06
II	0	62·5	0·00	0·00
	20	65·0	64·5	1·64
	35	70·0	70·1	1·92
	48	68·2	78·7	2·10
	78	69·7	88·7	2·42
	115	70·6	94·7	2·62
	153	77·5	91·4	2·77
	198	77·7	94·7	2·88
	253	80·0	94·5	2·96
	285	84·0	94·4	3·10

The total quantity of urea carbon synthesized during the experiments was obtained by multiplying the rise in plasma urea-carbon concentration by the 'urea space' of the cat. The 'urea space', i.e. the total volume of water in which the urea of the body is dissolved, has been found to be 65 % of the body weight of the cat (Kornberg *et al.* 1951*b*). Table 3 shows that the ratio

$$\frac{\text{carbon incorporated into urea}}{\text{carbon incorporated into urea} + \text{carbon expired as } CO_2}$$

was 0·037 or 0·038. Comparison of the distribution of radioactivity in urea and expired CO_2 show that the ratio

$$\frac{^{14}\text{C-urea synth. from labelled bicarbonate}}{^{14}\text{C-urea synth. from labelled bicarbonate} + {}^{14}CO_2 \text{ expired}}$$

was also 0·037 or 0·038. This means that within the experimental error of the average values (± 2 %) the urea carbon synthesized during the experiments must have been derived solely from carbon in equilibrium with blood bicarbonate, and confirms the results obtained *in vitro* by Grisolia & Cohen (1948), and *in vivo* by Mackenzie & du Vigneaud (1948), Sprinson (1949), and Armstrong & Zbarsky (1949).

DISCUSSION

Rate of expiration of $^{14}CO_2$

When ^{14}C-labelled sodium bicarbonate or carbonate is injected intraperitoneally into mice or rats, ^{14}C is rapidly expired as $^{14}CO_2$. Skipper *et al.* (1949), Skipper, Nolan *et al.* (1951), Skipper, Bennett *et al.* (1951) using mice, recovered 96 % of the injected dose in this form within 24 hr., 92·8 % having been expired within the first hour and over 50 % in the first 10 min. The total elimination in rats is very similar to that in mice, recoveries of 93–98 % being recorded (Armstrong & Zbarsky, 1949; Gould *et al.* 1949; Greenberg & Winnick, 1949) but the rate of expiration is slightly lower, 50 % of the injected ^{14}C appearing as $^{14}CO_2$ in the first 18 min. (Gould *et al.* 1949). The experiments described in the present paper show that in cats only 50 % of the injected ^{14}C is expired in the first 30 min., and that the total isotope expired in 5 hr. is 79–81 % of that injected. It is of interest that both the rate and total output of $^{14}CO_2$ was less in cats than in mice and rats, even though the material was injected intravenously into the cats, in contrast to the intraperitoneal injections

Table 3. *The source of the carbon of urea*

Cat no.	I	II
Body wt. (kg.)	1·55	1·83
Urea 'space' (ml.)	1010	1190
Blood-urea concentration (mg./100 ml.):		
At start	31·0	62·5
At end	50·1	84·0
(*A*) Total urea synthesized (mg. carbon)	44·0	59·0
(*B*) Total respiratory CO_2 output (mg. carbon)	1120	1500
$\frac{(A)}{(A)+(B)}$	0·037	0·038
(*A'*) ^{14}C fixed as urea (counts/min.)	15 600	21 700
(*B'*) $^{14}CO_2$ expired (counts/min.)	411 000	547 000
$\frac{(A')}{(A')+(B')}$	0·037	0·038

with the smaller animals. The rate of excretion of $^{14}CO_2$ after intravenous injection of labelled sodium bicarbonate can be formulated as an exponential equation with three components; at no time does it correspond to the first-order reaction described by Gould *et al.* (1949) after intraperitoneal injection of labelled bicarbonate into rats.

The 'bicarbonate pool'

The data of Gould *et al.* (1949) show that the specific activity of the expired $^{14}CO_2$ in their experiments falls at a uniform rate for the first 60 min., and that the initial specific activity obtained by extrapolation of the curve to zero time indicates a 'bicarbonate pool' of 2 m-moles/100 g. rat. As the total acid-volatile CO_2 in the extracellular and intracellular fluid of an animal of this size amounts to only 1 m-mole, the authors stated that 'the $C^{14}O_2$ must have rapidly entered into mobile equilibrium with 1 m-mole of CO_2 elsewhere in the body. One possibility is rapid exchange with the CO_2 of bone, and another is incorporation into dicarboxylic and tricarboxylic acids.' This conclusion and the earlier one of Brues & Buchanan (1948) that the total mass of carbon in mobile equilibrium with CO_2 in the body is greater than the total amount of CO_2 and bicarbonate in solution, is confirmed by our present findings, which provide evidence for the existence of exchange mechanisms between blood and tissue 'total CO_2'. After injection of labelled bicarbonate, ^{14}C is taken up into the tissues at varying rates from blood bicarbonate of initially very high radioactivity. The ^{14}C content of any tissue reaches a maximum when the specific activities of blood and tissue 'total CO_2' become nearly equal.

In these early stages of the experiments the distribution of ^{14}C amongst the blood and tissues will thus be in the order of their rates of CO_2 exchange. The 'fixed' isotope is released again into the blood at rates governed by many factors, such as the 'total CO_2' content, diffusion constants, blood supply, and metabolic activity of the tissue. The concentration of 'total CO_2' in cat blood is approx. 25 mM and a 2 kg. cat thus contains about 45 mg. carbon as acid-volatile CO_2 in its blood. Since, however, the rate of expiration of CO_2 is of the order of 90 mg. carbon/15 min. an amount of carbon equivalent to the total amount in the blood is expired about every 7 min. This rapid removal through the lungs of ^{14}C from the blood will preferentially deplete of their isotope content those tissues with the highest rates of exchange of CO_2, so that after 4 hr. the distribution in the tissues of the ^{14}C still remaining in the cat is, in general, in inverse order of these exchange rates (Table 1, cf. Skipper, Nolan *et al.* 1951). Of the ^{14}C injected as labelled bicarbonate in the present experiments, after 4 hr. only about 1% could be accounted for as the residual labelled bicarbonate of the blood, muscles and viscera, but approx. 6% was 'fixed' in bone. This large amount of incorporation is mainly due to the high 'total CO_2' content of bone. These facts present striking evidence for the occurrence of CO_2 exchange mechanisms in the body.

Further evidence is provided by the data on the expiration of $^{14}CO_2$ from the cat. As already stated, the graph of the percentage of injected ^{14}C expired against time is an exponential curve with three components (Fig. 3). These have half-lives of 2·85, 29·6 and 582 min. respectively, and, though the detailed nature of the three components is uncertain, they show that the 'bicarbonate pool' is certainly not homogeneous. This conclusion is also indicated when attempts are made to calculate the 'bicarbonate pool' of the cat by the procedure of Gould *et al.* (1949). The semi-logarithmic plot of specific activity against time (Fig. 4) is a smooth curve in each experiment, which at no time becomes linear. Attempts to calculate the 'bicarbonate pool' by dividing the ^{14}C remaining in the cat at any point by the ^{14}C-bicarbonate concentration of the blood at that point give a steadily rising 'pool' which after some time exceeds the body weight of the animal. The underlying assumption of this procedure, that all the ^{14}C remaining in the cat at any time is rapidly miscible with, and has the same specific activity as, blood bicarbonate, must therefore be erroneous, and 'fixation' of ^{14}C in some form not rapidly miscible with blood bicarbonate must therefore have occurred. This is supported by the fact that in the long-term experiments with mice and rats the 'total CO_2' of bone and other tissues of low metabolic activity (cf. Skipper *et al.* 1949; Skipper, Nolan *et al.* 1951; Neuberger, Perrone & Slack, 1951) have specific activities much higher than that of blood.

The synthesis of urea

Recent studies on the breakdown of labelled urea in mice (Leifer *et al.* 1948; Skipper, Bennett *et al.* 1951) and cats (Davies & Kornberg, 1950; Kornberg *et al.* 1951*b*) have shown that urea is not a stable end product of nitrogen metabolism, but is broken down in the body: after intravenous injection of ^{14}C-labelled urea into cats, approx. 2% of the isotope was expired in 5 hr. as $^{14}CO_2$. This slow evolution of CO_2 from urea may be likened to the processes occurring in a 'pool' with a low rate of CO_2 exchange, and the mechanisms of incorporation of ^{14}C into urea from ^{14}C-labelled bicarbonate should thus be similar to those postulated above. After 5 hr., 3·1% of the ^{14}C-labelled bicarbonate is present as urea carbon, but though the rate of urea synthesis was uniform throughout the experiments (Table 2), 50% of this incorporation occurred in the first 30 min. Most of this 'fixation' of ^{14}C thus took place when the radioactivity of blood bicarbonate was very high, the rate of release of 'fixed' ^{14}C being

governed by the rate of breakdown of urea. These facts thus fully support the views expressed above on the 'fixation' of ^{14}C in other tissues of the body.

It was first shown by Grisolia & Cohen (1948) *in vitro* and by Mackenzie & du Vigneaud (1948) *in vivo* that the specific activities of bicarbonate carbon and urea carbon are the same, and that the urea carbon is, within the experimental error, derived wholly from CO_2. The present findings (Table 3) confirm this view; this means that in our experiments with fasted cats less than 2% of the urea synthesized was formed from precursors other than labelled bicarbonate, such as arginine derived from the diet or from protein breakdown.

SUMMARY

1. Apparatus and techniques are described for *in vivo* studies of the metabolism of ^{14}C-labelled bicarbonate in the cat.

2. The rate of expiration of $^{14}CO_2$ after intravenous injection of $NaH^{14}CO_3$ is initially very rapid, 50% being expired in the first 30 min., the total expired in 5 hr. being 79–81% of the injected ^{14}C. The rate of expiration can be represented by an exponential equation with three components of half-lives 2·85, 29·6 and 582 min. respectively.

3. Of the ^{14}C injected, 3·1% was incorporated into urea in 5 hr., 50% of this incorporation occurring in the first 30 min. Within the experimental error (±2%) all the urea carbon synthesized was derived from carbon in rapid equilibrium with blood bicarbonate.

4. After 4 hr. approximately 6% of the injected ^{14}C was present in bone, the carbon of which exchanges relatively slowly with blood bicarbonate. The blood, muscles and viscera contained approximately 1% of the isotope at that time.

5. These results show that the bicarbonate pool is not homogeneous, but consists of several component CO_2-exchange mechanisms operating at widely differing rates.

We wish to thank Prof. H. A. Krebs, F.R.S., for his interest and advice, Mr T. Smith for technical assistance, Dr Q. H. Gibson and Mrs M. Davison for details of the design and use of the vacuum transfer apparatus, Prof. S. Gurin for advice on the preparation of solid samples for radio-assay, and Mr J. F. Scott for mathematical assistance. One of us (H.L.K.) wishes to acknowledge the receipt of a grant from the Medical Research Council.

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Studies on the Metabolism of Progesterone and Related Steroids *in vitro*

2. FACTORS INFLUENCING THE METABOLISM OF PREGNANE-3α:20α-DIOL BY RAT-LIVER HOMOGENATES, AND THE INVESTIGATION OF THE PRODUCTS OF METABOLISM

By J. K. GRANT
Department of Biochemistry, University of Edinburgh

(*Received* 11 *October* 1951)

In the first paper of this series (Grant & Marrian, 1950) experiments were described which showed that rat and rabbit liver preparations were able to metabolize pregnane-3α:20α-diol (pregnanediol) to unknown products *in vitro*. No metabolism occurred under anaerobic conditions unless a hydrogen acceptor (methylene blue) was added. The metabolism was uninfluenced by cyanide, and the evidence was compatible with the view that the agent in liver responsible for the metabolism of pregnanediol was a dehydrogenase. The possibility that more than one enzyme was involved was not excluded.

The work has now been extended with rat-liver homogenates, which have been employed in an investigation of factors influencing the metabolism of pregnanediol. The ultimate aim of these experiments was to obtain a system of optimum activity which could be used in an attempt to isolate products of metabolism in a large scale experiment.

It has been shown that the metabolism of pregnanediol by liver homogenates is increased by coenzyme I. In attempts to obtain the product or products of metabolism in large scale experiments pregnan-3α-ol-20-one was isolated, but in amounts only sufficient to account for approximately 10% of the metabolized pregnanediol.

EXPERIMENTAL

Materials and methods

Riboflavin and aneurin pyrophosphate were obtained from Roche Products Ltd.; adenosinetriphosphoric acid (ATP) was a gift from Dr H. Bergel, glutathione from Dr A. P. Meiklejohn and adenosinemonophosphoric acid (AMP) from Dr I. D. E. Storey. Coenzyme I (Co I) was prepared in the laboratory by the method of Le Page (1949), and its purity as determined spectrophotometrically (Le Page, 1947) was 71%. The weights and concentrations of Co I referred to are calculated as pure material unless otherwise stated. Pregnanediol dihemisuccinate (PDHS) was prepared as already described (Grant & Marrian, 1950). The difficulty previously experienced with PDHS tending to come out of solution was not found with later batches of this compound using the more concentrated phosphate buffer described below.

The standard medium used throughout this investigation was as follows (final concentrations): 0·114 M-NaCl, 0·046 M-KCl, 0·001 M-$MgSO_4.7H_2O$ and 0·056 M-potassium phosphate; pH 7·4. This medium, which will be referred to as the 'saline', differs from the calcium-free phosphate saline of Krebs & Eggleston (1940) used hitherto in the higher concentration of phosphate buffer. This increase in concentration was found desirable to maintain the pH during incubation with the concentration of homogenate used. No significant difference in results was observed when Na^+ in the saline was replaced by K^+ in order to obtain a medium more truly representative of intracellular fluid. As alteration in the concentration of K^+ appears to have a slightly adverse effect on the solubility of PDHS in saline, the Krebs-Eggleston type saline was retained although it admittedly does not resemble the inorganic pattern of intracellular fluid.

Homogenates were prepared from the livers of well fed female or male rats of the Wistar strain, approx. 1 year old. It was demonstrated in separate experiments (unpublished) that the sex of the animal did not influence the results. The rats were killed by dislocation of vertebrae in the neck. The livers were rapidly removed and chilled in crushed ice. After chopping roughly with scissors 3·5 g. liver were weighed into glass homogenizers (Potter & Elvehjem, 1936) standing in crushed ice, and ground with 6 ml. ice-cold water (unless otherwise stated) for approx. 1 min. with four passages of the pestle. The final homogenate was diluted to 10 ml. and kept at 0° until pipetted into incubation flasks.

The standard reaction mixture for 'incubation' experiments consisted of 2·5 ml. saline containing about 1 mg. pregnanediol as PDHS, and 1 ml. homogenate (giving a final concentration of 10% (wet wt./vol.) tissue). Unless otherwise stated, additions to this reaction mixture were made by weighing the substances concerned on a torsion balance, and adding the solid directly to the incubation flask. Freshly distilled pyruvic acid was diluted to suitable concentration, and carefully neutralized before adding to the saline.

In the quantitative experiments the procedure already described (Grant & Marrian, 1950) was followed. Incubations were in O_2 for 2 hr. at 37°. For 'controls' incubated reaction mixtures containing no PDHS were poured into 2·5 ml. PDHS saline and immediately worked up. 'Blank' values were found to be invariably low and very consistent. They were therefore not determined as a routine,

but were checked occasionally. A mean 'blank' value of 0·025 mg. 'apparent pregnanediol' was used to correct the values found in 'incubation' and 'control' experiments.

Decreased metabolism of pregnanediol observed when using rat-liver homogenates

The results of typical experiments using rat-liver homogenates are shown in Fig. 1 (bars *A* and *B*). The lower activity of liver homogenates in metabolizing pregnanediol will be evident from the fact that with these preparations over 80% of the pregnanediol originally present was recovered, as

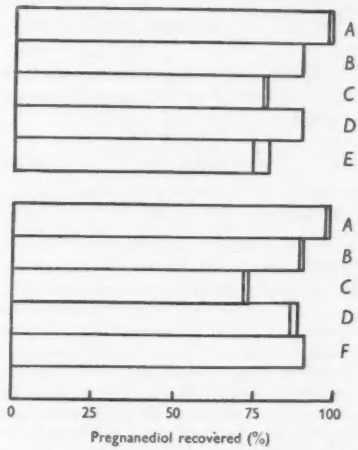

Fig. 1. Incubation of rat-liver homogenate with PDHS. Reaction mixture (*RM*) = about 1·6 mg. PDHS in 2·5 ml. saline + 1 ml. homogenate. Pregnanediol recovered, *A*, from 'controls'; after incubation of the following: *B*, *RM*; *C*, *RM* + 8×10^{-3}M-nicotinamide; *D*, *RM* using homogenate pre-incubated 0·5 hr.; *E*, *RM* using homogenate pre-incubated 0·5 hr. with added nicotinamide (final concn. 8×10^{-3}M); *F*, as *E* but nicotinamide added to homogenate after 0·5 hr. pre-incubation without nicotinamide.

In all figures two lines at ends of bars indicate two values, single lines duplicates.

compared with the recovery of only 50–60% pregnanediol incubated under the same conditions with rat-liver slices (Grant & Marrian, 1950). A similar loss of activity on mincing liver has been observed in metabolic studies with oestrogens (Coppedge, Segaloff & Sarrett, 1950) and with testosterone (Sweat & Samuels, 1948), and has been attributed by these workers to the rapid destruction of Co I in preparations of broken cells from liver and other organs (Mann & Quastel, 1941). The pH of 7·4 used in the various liver-steroid metabolism experiments reported by others and used in the present investigation is close to the maxima of 7·2 (Spaulding & Graham, 1947) and 7·5 (Handler & Klein, 1942) which have been reported for the nucleosidase concerned in the destruction of Co I. This destruction is, however, inhibited by nicotinamide (Mann & Quastel, 1941; Handler & Klein, 1942).

Effect of preparing homogenates in nicotinamide. As it seemed likely that the reduced activity of rat-liver homogenates could be accounted for by the destruction of Co I in the homogenate, attempts were made to protect the Co I originally present in the liver by adding nicotinamide. Homogenates were prepared for each experiment from a single rat liver as follows: (*a*) in saline; (*b*) in saline containing nicotinamide sufficient to provide a concentration of 0·2% in the final reaction mixture; (*c*) a portion of homogenate (*a*) was incubated 0·5 hr. at 37°, solid nicotinamide was then added to provide a final concentration of 0·2% (w/v) before use in PDHS experiments; (*d*) a portion of homogenate (*b*) was also incubated 0·5 hr. at 37° before use in the PDHS experiments. The results of experiments with these homogenates are shown in Fig. 1. It may be observed that there is an increase in metabolism of pregnanediol when using homogenates prepared in nicotinamide (bar *C*). Nicotinamide itself is not responsible for this increase since it has no effect when added to the homogenates which have been pre-incubated for 0·5 hr. (bar *F*). Homogenates which have been prepared in nicotinamide retain their higher activity on 0·5 hr. pre-incubation (bar *E*) supporting the view that the nicotinamide protects the Co I originally present. The observation that 0·5 hr. pre-incubation of homogenates prepared without nicotinamide did not further decrease their activity in metabolizing pregnanediol (bar *D*) may find an explanation in the very rapid destruction of Co I, possibly before the pre-incubation started. Alternatively, if several pathways exist for the metabolism of pregnanediol by liver preparations and one or more is not Co I sensitive, it is conceivable that a limited direction of pregnanediol into such routes might mask the loss of activity due to decreasing concentration of Co I.

Effect of added Co I. The effect of various concentrations of added Co I on the metabolism of pregnanediol by rat-liver homogenates prepared in nicotinamide solution is shown in Fig. 2. From these results it appeared that rather high concentrations of Co I would be required to obtain the 50% metabolism previously observed with liver slices. This might be due to stimulation of metabolism by an impurity in the Co I preparation, rather than by the Co I itself. Alternatively, the concentration of nicotinamide used may have been inadequate to protect the added Co I. AMP is the chief impurity in the Co I preparation (Le Page, 1949), but when this substance in concentration of 4×10^{-3}M replaced Co I in the reaction mixture it had no effect on the

activity of the homogenate in metabolizing pregnanediol. In order to investigate the second possibility varying quantities of nicotinamide were added to the standard reaction mixture. When required, Co I was added (after the nicotinamide) to give a final concentration of $1{\cdot}5 \times 10^{-3}$M. It may be seen from the results shown in Fig. 3 that an optimum effect was obtained using $1{\cdot}5 \times 10^{-3}$M-Co I and 8×10^{-2}M-nicotinamide (bar *F*).

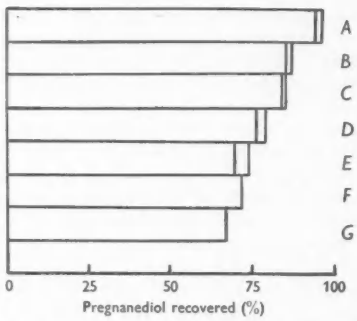

Fig. 2. Incubation of rat-liver homogenate with PDHS; influence of Co I on pregnanediol metabolism. Reaction mixture (*RM*) = about 1·6 mg. PDHS in 2·5 ml. saline + 1 ml. homogenate + 8×10^{-3}M-nicotinamide. Pregnanediol recovered is shown as follows: *A*, from 'controls'; *B*, after incubation of *RM*; after incubation of *RM* with Co I; *C*, 7×10^{-4}M-Co I; *D*, $1{\cdot}3 \times 10^{-3}$M-Co I; *E*, 2×10^{-3}M-Co I; *F*, $2{\cdot}6 \times 10^{-3}$M-Co I; *G*, $3{\cdot}2 \times 10^{-3}$M-Co I.

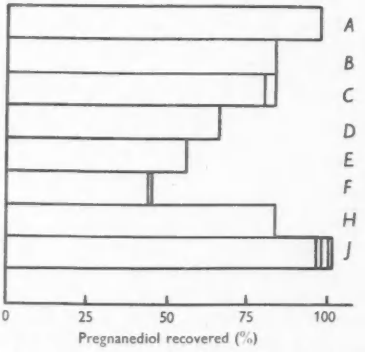

Fig. 3. Incubation of rat-liver homogenate with PDHS; protection of added Co I by nicotinamide. Reaction mixture (*RM*) = about 1·6 mg. PDHS in 2·5 ml. saline + 1 ml. homogenate. Pregnanediol recovered is shown as follows; *A*, from 'controls'; after incubation of the following: *B*, *RM*; *C*, *RM* + 8×10^{-3}M-nicotinamide; *D*, *RM* + 8×10^{-3}M-nicotinamide + $1{\cdot}5 \times 10^{-3}$M-Co I; *E*, *RM* + 4×10^{-2}M-nicotinamide + $1{\cdot}5 \times 10^{-3}$M-Co I; *F*, *RM* + 8×10^{-2}M-nicotinamide + $1{\cdot}5 \times 10^{-3}$M-Co I; *G*, *RM* + 0·12M-nicotinamide + $1{\cdot}5 \times 10^{-3}$M-Co I; *H*, *RM* + 8×10^{-2}M-nicotinamide; *J*, *RM* without homogenate + 8×10^{-2}M-nicotinamide + $1{\cdot}5 \times 10^{-3}$M-Co I.

This amount of nicotinamide had no significant effect in the absence of Co I (bar *H*). Incubation of PDHS with Co I and nicotinamide without liver homogenate did not result in any disappearance of pregnanediol (bar *J*).

Effect of other added substances. The following substances were tested individually in the standard reaction mixture containing $1{\cdot}6 \times 10^{-2}$M-nicotinamide and were found to be without effect on the metabolism of pregnanediol-ascorbic acid (7×10^{-9}M), glutathione (3×10^{-3}M), cocarboxylase (3×10^{-4}M), riboflavin (4×10^{-4}M), pyruvate (10^{-2}M), citrate (10^{-2}M), glucose (2×10^{-2}M), ATP (10^{-2}M).

The addition of 0·025M-semicarbazide in an attempt to trap ketonic metabolic products was also without effect.

Investigation of the products of metabolism

Experiments on a larger scale were arranged to permit the attempts to isolate the product or products of metabolism of pregnanediol by rat-liver homogenates.

For this purpose pregnanediol was purified before conversion to PDHS by recrystallization from benzene and ethanol yielding a product which melted at 237–238°. (All melting points are corrected, and unless otherwise stated were determined on a Kofler-type apparatus; Klyne & Rankeillor, 1947.) The PDHS prepared in the usual way (Grant & Marrian, 1950) from this pregnanediol melted at 147–148°. (Found (material dried at 80° for 1 hr. *in vacuo* over P_2O_5): C, 67·1; H, 8·5. Calc. for $C_{29}H_{44}O_8$: C, 67·2; H, 8·5%.)

The rat-liver homogenates were prepared in small batches in chilled glass homogenizers as described above. The Atomix Blender, with high-speed rotating blades, was not used for the preparation of the larger quantities of homogenate required since its use results in excessive frothing, the temperature is difficult to control and it has been reported by Stern & Bird (1949) and Lambden (1950) that the similar Waring Blendor is capable of inactivating certain enzymes.

The large scale experiment was conducted in five parts, a total of 500 mg. pregnanediol as PDHS being incubated with rat-liver homogenate.

The following is a description of a typical experiment. PDHS (163 mg. containing 100 mg. pregnanediol) was dissolved in 245 ml. saline containing 3·5 g. nicotinamide and 0·5 g. Co I. After checking that the pH was 7·4 the solution was diluted to 250 ml. Two 2·5 ml. volumes were removed for pregnanediol analyses, the remainder was cooled in ice and water.

Fresh rat liver (70 g.) was minced roughly with scissors, mixed and divided into two parts. One part was homogenized at 0° in seven 5 g. portions with seven 15 ml. volumes of the chilled PDHS saline. These homogenate reaction mixtures were pooled, thoroughly mixed with the bulk of the PDHS saline and distributed in ten 100 ml. conical flasks. The flasks were filled with O_2, stoppered, and shaken in a bath for 2 hr. at 37°.

The remaining 35 g. liver were treated in an identical fashion using saline which contained no PDHS and no CoI to provide a liver control experiment.

After incubation, the contents of the flasks were pooled, diluted to 300 ml. and two 3·0 ml. volumes were removed for pregnanediol analyses. The bulk of the mixture was poured into 1500 ml. cold dry acetone, thoroughly mixed in an Atomix Blender and filtered. The filter cake was washed with two 250 ml. volumes of acetone using the blender for efficient mixing. The filtrate was distilled under reduced pressure until the volume was 190 ml. The pH was found to be 7·0. This aqueous residue was transferred to large continuous liquid/liquid extractors with 50 ml. hot ethanol and extracted with ether (250 ml.) for 6 hr. adding 10 ml. ethanol to the aqueous phase after 2 and 4 hr. The pale-yellow ether extracts were washed twice with 50 ml. volumes 0·1 N-HCl, with 50 ml. 5% (w/v) $NaHCO_3$ and finally with 2 × 50 ml. water. Emulsions formed during the early washings were broken by the addition of solid NaCl. The ether was evaporated to give 1·3 g. dark-brown gum in the PDHS experiment and 1·0 g. in the liver control experiment. Little significance was attached to these weights, since the gums appeared to retain solvent which was lost slowly in the vacuum desiccator. For the separation of ketonic material the ether-soluble neutral fractions, dried in a vacuum desiccator over $CaCl_2$ for 24 hr., were heated under reflux condenser for 1 hr. on a boiling-water bath with 0·5 g. Girard's Reagent T (Girard & Sandulesco, 1936), 1·5 g. glacial acetic acid and 10 ml. 95% ethanol. After cooling, 88 ml. water, 30 g. crushed ice and 11·5 ml. 2N-NaOH were added and the whole was extracted with 100 ml. and three 50 ml. volumes of ether. Troublesome emulsions usually formed during this extraction. The combined ether extracts were washed with 25 ml. 5% $NaHCO_3$ and twice with 25 ml. water. A small amount of white solid which separated at the interface between ether and water during the washing was added to the ether phase. The washed ether was taken to dryness (non-ketonic fraction). 16 ml. conc. HCl were added to the ether-washed aqueous phase to hydrolyse Girard complexes. After standing overnight ketones were extracted with 100 ml. and three 50 ml. volumes of ether. The combined ether extracts were washed and evaporated as before to give the ketonic fraction.

On account of the troublesome emulsions formed during extraction of the non-ketonic fraction in all experiments it was thought that this fraction was likely to have retained ketonic material. It was therefore subjected to a second Girard separation in each case. Ketonic and non-ketonic fractions were subsequently pooled for further examination.

The quantities of pregnanediol recovered after incubation in the five experiments, calculated as percentages of the amount of pregnanediol originally present, were 44, 48, 49, 59 and 47%. These figures indicate the extent of metabolism in each experiment.

The purity of the pregnanediol used in these experiments was checked in a pregnanediol control experiment as follows: 70 g. rat liver were homogenized and incubated in saline without PDHS, and an ether-soluble neutral fraction was prepared from the incubated material as already described. 200 mg. pregnanediol were then added and the mixture was subjected to the Girard separations and other procedures applied in the PDHS experiments.

The pooled ketonic and non-ketonic fractions from all experiments were brown gums. The weights of the various fractions are shown in Table 1.

Table 1. *Weights of non-ketonic and ketonic fractions isolated from large scale rat-liver homogenate experiments*

	Liver incubated (g.)	Pregnanediol incubated (g.)	Fractions isolated	
			Non-ketonic (g.)	Ketonic (g.)
PDHS experiment	175	0·5	4·175	0·208
Liver control	175	0	3·760	0·132
Pregnanediol control	70	0·2	0·964	0·074

Each ketonic fraction was subjected to one further Girard separation yielding final ketonic fractions as follows: PDHS experiment, 57 mg.; liver control experiment, 13 mg.; pregnanediol control experiment, 12 mg.

Pregnan-3α-ol-20-one from the ketonic products of metabolism of pregnanediol by rat-liver homogenates

The final ketonic fraction in each case was dissolved in 10 ml. hexane and poured on to a column of 5 mm. diameter packed with 1 g. Al_2O_3 (Peter Spence and Co., Widnes, Type H, dried *in vacuo* at 100°, Activity II (Brockmann & Schodder, 1941)). The columns were eluted successively with four or five 10 ml. portions of each of the following anhydrous solvents: hexane, benzene, ether and acetone.

In the case of the ketonic fraction isolated from the PDHS experiment crystalline residues were obtained by evaporation of the benzene eluates (26 mg.). No crystalline material was obtained in the case of the liver control or pregnanediol control experiments.

An unsuccessful attempt was made to prepare a solid semicarbazone derivative from the gum (9 mg.) eluted by hexane in the case of the PDHS experiment.

The crystalline material from the benzene eluates (PDHS experiment) dissolved in 10 ml. hexane leaving a small amount of insoluble gum. The hexane solution was poured on to a column of Al_2O_3 prepared as already described, and the column was eluted successively with four 10 ml. portions of hexane, twenty-two 10 ml. portions of benzene, six 10 ml. portions of 3:1 benzene:ether and, finally, six 10 ml. portions of ether. Evaporation of the benzene eluates gave 21·5 mg. white crystalline solid which melted at 148–150°.

After recrystallization from hexane and drying at 80° for 24 hr. *in vacuo* over P_2O_5 the substance melted at 148–149°. Mixed with authentic pregnan-3α-ol-20-one (m.p. 147–149°) the melting point was 147–148°. $[\alpha]_D^{14°} = +103·6° \pm 0·5$ in ethanol ($c = 0·965$). Marrian & Gough (1946) reported $[\alpha]_D^{15°} = +107°$ in ethanol for authentic pregnan-3α-ol-20-one.

By treatment with semicarbazide hydrochloride and sodium acetate for 3 days at room temperature in aqueous

ethanolic solution, a product was obtained which after one crystallization from absolute ethanol and one from 80% (v/v) aqueous ethanol melted at 249–251°; mixed with authentic pregnan-3α-ol-20-one semicarbazone (m.p. 248–250°) the melting point was 249–250°.

Investigation of non-ketonic fractions

Hexane-insoluble material was prepared from non-ketonic fractions by repeated leaching with boiling hexane and chilling the leachings for several hours in the refrigerator. In this way the non-ketonic fraction from the PDHS experiment and from the pregnanediol control experiment gave material which after recrystallization was identified as pregnane-3α:20α-diol. Crystalline pregnanediol (62 mg.) was obtained from the former experiment, 42 mg. from the latter. No hexane-insoluble material was obtained from the liver control experiment.

Hexane-soluble material in each experiment was dissolved in 50 ml. hexane and poured on to columns of 25 mm. diameter packed with 50 g. Al_2O_3. The columns were eluted successively with the following solvents: hexane, benzene, ether, acetone, ethanol and methanol. Cholesterol was isolated and identified from the acetone eluates from all experiments. The gums remaining after evaporation of the other solvents resisted all attempts at crystallization.

Thus no crystalline material except pregnanediol and cholesterol was obtained from the non-ketonic fractions.

DISCUSSION

The evidence presented in this paper shows that Co I is required by the enzyme system or systems involved in the *in vitro* metabolism of pregnanediol by rat liver.

In the investigation of the products of metabolism approximately half of the pregnanediol incubated with the liver was metabolized in some way. Evidence was presented in a previous paper (Grant & Marrian, 1950) to show that the pregnanediol which disappears cannot be accounted for by the formation of conjugated substances, nor can the pregnanediol which disappears be accounted for by absorption on the relatively large weight of liver tissue, since the treatment with potassium hydroxide at 100° before extraction of the pregnanediol dissolves the tissue in addition to hydrolysing any unchanged dihemisuccinate, and added pregnanediol has always been recovered in good yield from these experiments carried out in nitrogen in place of oxygen. In the experiments now reported a little over 10% of the pregnanediol metabolized was accounted for as crystalline ketonic material, which appeared to consist mainly of pregnan-3α-ol-20-one. No evidence was obtained regarding the fate of the main part of the pregnanediol which disappeared.

Sweat, Samuels & Lumry (1950) have prepared an enzyme from steer liver which catalyses the oxidation of testosterone to androst-4-ene-3:17-dione. Co I was found to be necessary as a hydrogen acceptor in the system but marked reduction of the cytochrome *c* concentrations of the preparation did not affect its activity. The presence of this enzyme in the livers of a variety of species, including the rat, has also been demonstrated (Samuels, Sweat, Levedahl, Pottner & Helmreich, 1950). The possibility that the same enzyme may be involved in the oxidation of pregnane-3α:20α-diol to pregnan-3α-ol-20-one cannot be excluded, since Samuels and his co-workers have not investigated the effect of their enzyme on 20-hydroxy steroids (Samuels, 1951). Both the 'testosterone' and the 'pregnanediol' enzyme require Co I, and both appear to be capable of functioning in systems independent of cytochrome *c*.

SUMMARY

1. It has been shown that coenzyme I is required by an enzyme system or systems in rat-liver homogenates, which are able to metabolize pregnane-3α:20α-diol.

2. In a large scale experiment 50% of the pregnane-3α:20α-diol incubated with rat-liver homogenate was metabolized. Of this amount 10% was recovered as crystalline pregnan-3α-ol-20-one. No other crystalline products of the metabolism of pregnanediol were obtained.

The author is indebted to Prof. G. F. Marrian, F.R.S., for constant interest and advice in this work; to Dr J. W. Minnis for micro-analyses and to Mrs S. M. Atherden for skilled technical assistance. The expenses of this work were largely defrayed out of a grant from the Medical Research Council to Prof. Marrian.

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Enzymic Reduction of 2:4-Dinitrophenol by Rat-tissue Homogenates

By V. H. PARKER
Medical Research Council Unit for Research in Toxicology, Serum Research Institute, Carshalton, Surrey

(*Received* 3 *October* 1951)

During an investigation into the toxic properties of 3:5-dinitro-*o*-cresol (DNOC) and 2:4-dinitrophenol (DNP) the breakdown of these substances by animal tissues *in vitro* was studied. Dinitrophenol and dinitro-*o*-cresol have been studied as inhibitors of phosphorylation in various enzyme systems and have been shown to affect the metabolism of tissues *in vitro*, but little has been reported of the effect of such systems on the dinitrophenol molecule. There is some information on the metabolic products of dinitrophenol by Magne, Mayer & Plantefol (1932) who state that the excretion products in the urine after administration of dinitrophenol to man and dogs were unchanged dinitrophenol and 2-amino-4-nitrophenol. Later, Greville & Stern (1935) demonstrated that 2-amino-4-nitrophenol was the main product after dinitrophenol had been in contact with a bacterial succinic dehydrogenase system.

The present studies were undertaken in order to ascertain the possible metabolic products of DNP and DNOC, and the organs mainly responsible for the metabolic change. As three of the amino derivatives of dinitrophenol were readily obtained, most of the following experiments were done with this compound in preference to DNOC.

EXPERIMENTAL

Preparations. Rats were killed by a blow on the head and immediately afterwards their throats were cut and the bodies bled out.

Liver homogenate. The tissue was removed, weighed and homogenized in 15 ml. of 0·06 M-phosphate buffer, pH 7·4, for 1 min. Phosphate buffer was then added to make the final proportions of 3 parts of buffer to 1 part of tissue. Unless otherwise stated the homogenate was used at once.

Liver extract[a]. These were made by grinding a known weight of liver with washed sand and phosphate buffer in a pestle and mortar. After centrifugation, the supernatant was removed and used in volumes equivalent to 2·5 g. of homogenized liver.

Liver brei. This was prepared by mincing liver in a domestic mincing machine.

Acetone-dried liver. This was prepared by homogenizing 25 g. liver in 100 ml. of ice-cold acetone. The mixture was centrifuged and the supernatant discarded. The residue was resuspended in 100 ml. cold acetone and recentrifuged. The solid residue was washed with 100 ml. cold ether. After centrifuging, the solid residue was spread to dry on filter paper. The dried powder was used at once.

Pieces of whole liver (approximately 0·5 g.) were freeze-dried in small bottles in a centrifugal freeze-dryer.

Homogenate experiments. For the study of the enzymic process, 8 ml. of homogenate were measured into a test tube. Inhibitors or activators also prepared in buffer solution were added at this stage. To this was added 4 ml. of dinitrophenol 0·5 mg./ml. prepared in buffer solution, pH 7·4. Finally, the volume was made up to 20 ml. with buffer solution giving a final concentration of dinitrophenol of $5{\cdot}4 \times 10^{-4}$ M. The contents of the tube were mixed by shaking and the tube placed in a water bath at 37°. A control tube was prepared each time containing the same concentration of dinitrophenol and inhibitor or activator in a total volume of 20 ml. of buffer solution. At intervals of 0·5 hr., 0·5 ml. samples were removed with a Krogh-Keys syringe pipette and analysed for dinitrophenol or aminonitrophenol according to the requirements of the experiment. The buffer solution, pH 7·4, was 0·06 M-phosphate containing 7×10^{-2} M-succinate.

Determination of DNP. Dinitrophenol was estimated by the method based on extraction of the alkali salt with methyl ethyl ketone followed by colorimetry (Parker, 1949).

Determination of aminonitrophenols. The proteins of 0·5 ml. homogenate were precipitated by the addition of 1 ml. 15% (w/v) trichloroacetic acid. The mixture was centrifuged, 1 ml. supernatant was mixed with 2 ml. water and 1 ml. 5 N-HCl. The solution was then cooled in ice water and placed in a dark cabinet. This was necessary because it was found that the diazonium compounds in solution were decomposed by daylight. Diazotization was carried out by the addition of 0·5 ml. 0·2% $NaNO_2$ solution, mixing and leaving in the dark for 10 min. Excess HNO_2 was removed by addition of 1 ml. 1% ammonium sulphamate solution. After a further 10 min. the diazonium compound was coupled with 2 ml. 0·5% *N*-1-naphthylethylenediamine prepared in 3 N-HCl. The tube was placed in a water bath at 37° and the colour developed for 30 min. At the end of this time, the optical density of the solution was measured at 550 mμ. on a Unicam D.G. spectrophotometer. Standard curves were prepared from the two isomeric aminonitrophenols.

Extraction of the ether-soluble amino compound from liver homogenates. Liver homogenate (50 g.) was prepared as above. DNP concentration used was 100 μg. DNP/ml. After incubation for 1 hr. at 37° the reaction was stopped and the protein precipitated by 15% trichloroacetic acid, cooled and centrifuged. The supernatant liquor was made just alkaline with NH_4OH and then just acid to litmus paper with acetic acid. The solution was then extracted three times with

redistilled ether. The combined ether extracts were dried and evaporated to dryness. The brownish residue was dissolved in a small amount of ethanol (1–2 ml.).

Chromatography. A chromatograph column was prepared in an all-glass apparatus using a sludge of ethanol and alumina (P. Spence and Sons Ltd. 778–780 Salisbury House, London, E.C. 2. Activated alumina, Type H). The ethanolic extract of liver homogenate was placed on the top of the column and the chromatogram developed with ethanol containing a little NH_3 (approximately 0·15 N). The rate of development was increased by the application of a small positive pressure to the top of the column. As a rule, three bands were seen moving separately down the column. The last band was eluted by changing the composition of the developing fluid to one of 90% ethanol containing 0·15 N-NH_3. Each component was collected separately as it appeared.

Paper-partition chromatography. A pencil line was drawn across a strip of Whatman filter paper no. 3 (25 × 3·5 cm.) at a distance of 4 cm. from one end, a second pencil line at 15·0 cm. from the first. A small quantity (10–30 μl.) of the ethanolic extract of liver homogenate was placed on the lower line and dried. With each chromatogram of the unknown substance a control was run consisting of a mixture of DNP and the isomeric amino compounds. A spot of this mixture was placed on the line on one side of the 'unknown' spot. The strip was suspended in a gas jar so that the end of the paper nearest to the spots of solutions was just dipping into the developing fluid. The gas jar was filled with N_2 by displacement of air and closed with a greased lid and development of the chromatogram allowed to take place by upward capillary attraction until the liquid front reached the second pencil line. At this stage the paper was removed and allowed to dry in the air. The developing fluid consisted of 20% benzene, 20% pentanol, 40% ethanol and 20% ammonia solution (sp.gr. 0·880). All percentages were v/v.

Spectroscopy. Some of the solutions obtained by chromatography were examined for ultraviolet absorption in the Unicam Quartz S.P. 500 spectrophotometer. The absorption curves were determined from 250 to 400 mμ. at a slit width of 0·1 mm.

All but two of the following experiments took place in buffered solutions at pH 7·4. The conditions for the two exceptions are mentioned in the text.

RESULTS

Characteristics of the enzyme preparation. The activity of the homogenates, measured as μg. DNP destroyed per ml. of solution in 1 hr. was at a maximum when the liver was prepared in phosphate buffers at pH values of 7·0–7·5.

The optimum temperature was approximately 37°. Heating the homogenate to 100° resulted in complete loss of activity, whilst maintaining the preparation at 60° for 10 min. resulted in a decrease in activity by 70%. There was no destruction of DNP when the homogenate was kept at 0°.

The activity of the preparation depended upon the length of time taken to homogenize the liver and upon the age of the homogenate. Preparations homogenized for periods of time varying from 30 sec. to 6 min. exhibited varying degrees of activity, the most active preparation being that which had been homogenized for 1 min. Experiments also showed that the homogenate was most active if used immediately after preparation. Left for periods of up to 2 hr. at 37° the homogenates lost 20% of their activity. From 2 to 4 hr. there was little further loss in activity.

Michaelis constant, K_m and effect of enzyme concentration. In order to determine the Michaelis constant for the reaction, it was necessary to remove samples from the homogenate at intervals of 5 min. The reciprocals of the amount of DNP destroyed in 5 min. ($1/v$) were plotted against the reciprocals of the concentration ($1/c$) as suggested by Lineweaver & Burk (1934). The Michaelis constant was derived from the slope of the line, K_m/v_m, and it was found to be $8{\cdot}5 \times 10^{-5}$ M-DNP (Fig. 1).

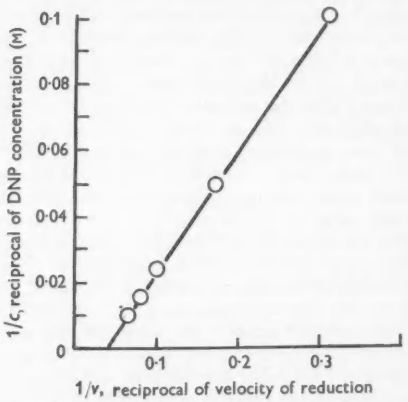

Fig. 1. Effect of DNP concentration within the range of $5{\cdot}4 \times 10^{-5}$ to $5{\cdot}4 \times 10^{-4}$ M on the amount of DNP destroyed by liver homogenates. v = moles DNP destroyed in 5 min.

Fig. 2 shows the result of varying the concentration of the homogenate. There is a linear relationship between the amount of enzyme present and the amount of DNP destroyed when not more than 80% of DNP is destroyed.

Comparison of homogenates with other preparations. When the activities of liver extract and liver brei were compared with the activity of an equivalent quantity of liver homogenate it was found that under identical conditions there was no difference between the three forms of preparations. In other experiments, acetone-dried liver and freeze-dried liver were compared with homogenates. Acetone-dried liver powder equivalent to 4 g. of fresh liver destroyed only 30% of DNP (initial concn. $5{\cdot}4 \times 10^{-4}$ M) in 1 hr. compared with 60% of DNP destroyed by 2·5 g. of homogenized liver in the same time. Whole liver freeze-dried had approximately

90 % of the activity of homogenized liver. Freeze-dried liver retained its initial activity in a refrigerator for two weeks.

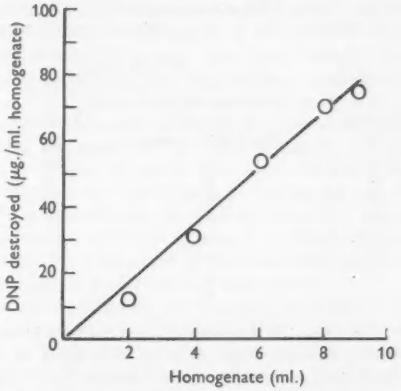

Fig. 2. Effect of enzyme concentration on amount of DNP destroyed. Enzyme concentration is expressed as ml. of liver homogenate in range of 2–9 ml. (15 g. liver homogenized and made up to a final volume of 60 ml.). Each solution analysed after 1 hr. at 37°. Initial DNP concentration $5{\cdot}4 \times 10^{-4}$ M.

An attempt was made to determine the active portion of the homogenate. An homogenate was divided into three portions *A*, *B* and *C*. *A* was incubated with DNP in the usual way. *B* was centrifuged

Table 1. *Activity of liver homogenate fractions*

(Preparation *A*, 2·5 g. liver homogenized, DNP added and volume adjusted to 20 ml. Preparation *B*, 2·5 g. liver homogenized in 10 ml. buffer solution, centrifuged, DNP added to supernatant fluid. Volume adjusted to 20 ml. Solid residue from *B* suspended in buffer solution, DNP added and volume adjusted to 20 ml. Preparation *C*, 2·5 g. liver homogenized in 10 ml. of buffer solution, entrifuged and supernatant fluid filtered through kieselguhr. Filtrate adjusted to 20 ml. after addition of DNP. DNP concentration, 100 μg./ml., pH 7·4.)

Preparation	Whole homogenate of *A*	Supernatant of *B*	Residue of *B*	Filtered supernatant of *C*
μg. DNP destroyed per ml. after 60 min.	56	41	13	8
Activity expressed as percentage activity of whole homogenate	100	73	23	15

at the top speed of an angle centrifuge and the supernatant fluid and solid residue incubated separately with DNP. *C* was also centrifuged, but the supernatant fluid was filtered through a layer of kieselguhr before assessment of its activity. Table 1 indicates that most of the activity resides in the opalescent supernatant fluid after centrifuging, but is lost by filtration.

Effect of enzyme inhibitors. The following compounds were examined for their effect on the activity of liver homogenates: potassium cyanide, *p*-chloromercuribenzoic acid (CMBA), iodoacetic acid, sodium pyrophosphate, sodium azide and sodium malonate. Table 2 shows that only cyanide inhibited the destruction of dinitrophenol.

Table 2. *Effect of inhibitors on destruction of DNP*

(For each test, 2·5 g. of liver were homogenized in 15 ml. of phosphate buffer, and 1 ml. of inhibitor added. After incubation at 37° for 30 min., DNP was added to a final concentration of $5{\cdot}4 \times 10^{-4}$ M and a total volume of 20 ml. Samples were removed for analysis at 30 and 60 min.)

Compound	Final concn. (M)	Inhibition (%)
Potassium cyanide	5×10^{-5}	32
	5×10^{-4}	90
	5×10^{-3}	100
Sodium azide	5×10^{-3}	8
Iodoacetic acid	5×10^{-3}	8
p-Chloromercuribenzoic acid	5×10^{-3}	0
Sodium pyrophosphate	5×10^{-2}	0
Sodium malonate	5×10^{-3}	0

Other factors influencing the breakdown of DNP. Greville & Stern (1935) and Westfall (1943) used a dehydrogenase system to demonstrate the reduction of nitro groups in dinitrophenol and trinitrotoluene. The effect of substrates for dehydrogenases was observed on the activity of liver homogenates in destroying DNP. It was found that their activity could be increased by almost 20 % when 7×10^{-2} M-succinate or lactate was added. The experiments reported above were carried out under aerobic conditions. Westfall demonstrated that the reduction of trinitrotoluene also occurred under anaerobic conditions. The rate of decomposition of DNP under aerobic and anaerobic conditions were compared. DNP was added to chilled liver homogenate in Thunberg tubes immersed in ice water. The DNP concentration was $5{\cdot}4 \times 10^{-4}$ M after adjustment to a known volume with phosphate buffer, pH 7·4. The tubes were evacuated and refilled with nitrogen three times in succession. These tubes as well as similar tubes, open to the atmosphere, containing the same amount of liver homogenate and DNP were incubated at 37° for 1 hr. It was found that the reaction took place at the same rate under aerobic and anaerobic conditions.

That the succinic oxidase system was not responsible for the reduction of dinitrophenol was demonstrated by the following experiment. Succinic oxidase was prepared by Slater's modification of Keilin & Hartree's method (Slater, 1949). The final preparation was such that 0·5 ml. of a 1 in 25 dilution

Table 3. *Enzymic activity of rat and rabbit tissues*

(Spleen, kidney, heart, intrascapular fat and brain tissues were removed from the freshly killed animal, weighed and homogenized in 3 parts of phosphate buffer. Liver and muscle, 2–3 g. weighed and homogenized in 3 parts of phosphate buffer. DNP added to a final concentration of $5{\cdot}4 \times 10^{-4}$ M. Blood, 5 ml. taken, DNP in phosphate added to a final concentration of $5{\cdot}4 \times 10^{-4}$ M-DNP. All homogenates incubated at 37° and analysed after 30 min. DNP destroyed calculated in terms of mg./100 g. wet wt. tissue in 30 min.)

	Rat tissue		Rabbit tissue	
	DNP destroyed in 30 min. (mg./100 g. tissue)	Activity (%)	DNP destroyed in 30 min. (mg./100 g. tissue)	Activity (%)
Liver	68	100	29	100
Spleen	40	59	0	0
Kidney	41	60	12	41
Heart	20	29	1	3
Intrascapular fat	33	47	—	—
Muscle	11	16	—	—
Brain	2	3	—	—
Blood	0	0	0	0

reduced 1ml. 10^{-5} M-methylene blue in 30 min. For the determination of its effect on DNP 1 ml. of the undiluted enzyme preparation was used. The buffer solution, pH 7·7, was 0·01 M-phosphate solution containing 0·03 M-sodium bicarbonate. The Thunberg tubes were filled with 5% carbon dioxide in nitrogen after removal of air. It was found that with DNP concentrations of $4{\cdot}5 \times 10^{-5}$ to $4{\cdot}5 \times 10^{-4}$ M no destruction of the nitro compound occurred.

Distribution of the enzymic activity. The activity of other tissues of the rat was examined by making homogenates from them and incubating with dinitrophenol under identical conditions.

Table 3 shows that the activity of the rabbit tissue is lower than that of the corresponding rat tissue. On the other hand, when injected into the living animal, DNP disappears from the blood stream of the rabbit within a few hours, whereas it remains in the blood of the rat for several days (Parker, Barnes & Denz, 1951).

Reduction of DNOC. Experiments were performed with rat homogenates in which DNOC was substituted for DNP. It was found that these homogenates could reduce DNOC and the rate of reduction was measured as rate of disappearance of DNOC. Various tissues had similar relative capacities of reducing DNOC as they had in reducing DNP. The product of DNOC was a diazotizable amine, but was not identified.

The reduction product of DNP

The experiments described above indicated that DNP was destroyed by liver homogenates by an enzymic process. The products of this reaction were sought. A homogenate which had been in contact with DNP for 1 hr. was taken. The reaction was stopped by addition of 5 ml. of 20% trichloroacetic acid to every 20 ml. of homogenate. A portion of the supernatant was diazotized and coupled with *N*-1-naphthylethylenediamine. A magenta dye was produced. On the assumption that only primary aromatic amines form dyes of this kind, it was decided that at least one of the metabolic products was such an amine. The possible amino derivatives of 2:4-dinitrophenol are 4-amino-2-nitrophenol, 2-amino-4-nitrophenol and 2:4-diaminophenol. The isomeric nitroamines can be separated from the diamine by simple ether extraction in acid solutions, and it was found that the greater part of the amino fraction could be extracted in this way.

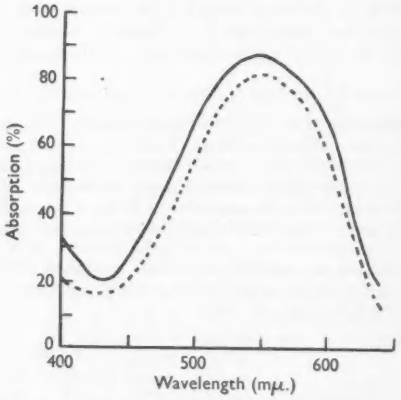

Fig. 3. Absorption curves of diazo dyes of aminonitrophenols. Aminonitrophenol diazotized (25 μg.) and coupled with *N*-1-naphthylethylenediamine in a final volume of 7·5 ml. ——, dye of 2-amino-4-nitrophenol; - - - - -, dye of 4-amino-2-nitrophenol.

The diazotization method could be used to estimate either of the two aminonitrophenols separately. But the diazo dyes that were produced did not differ sufficiently to enable one amine to be detected in the presence of the other. The characteristics of the absorption curves are given in Fig. 3.

It is seen that both dyes have very nearly the same intensity of colour and the maximum absorption occurs at the same wavelength. Magne *et al.* (1932) had detected 2-amino-4-nitrophenol in the freshly voided urine of workers exposed to DNP dust. Their method was a modification of the Derrien test in which the solution was diazotized, coupled with alkaline 2-naphthol and the resultant dye extracted into ether. After addition of acetic acid and zinc ions to the ethereal solution, the 2-amino-4-nitrophenol was identified by the production of a pink colour. Under the same conditions the 4-amino-2-nitro compound produced a yellow colour. The authors quoted above used this method for the examination of the liver of a dog which had been poisoned by DNP, and in this case they concluded that 4-amino-2-nitrophenol was present.

When the Derrien test was used to examine liver homogenates, a yellow colour was produced. This was an indication of the presence of 4-amino-2-nitrophenol, but was not conclusive evidence for the absence of the isomer. Excess of 4-amino-2-nitrophenol could hide the presence of smaller amounts of 2-amino-4-nitrophenol.

A mixture of the pure compounds was added to an alumina column. On development with ammoniated ethanol, a yellow band first appeared which very soon moved well ahead of the bulk of the coloured compounds remaining at the top of the column. This yellow compound was eluted and identified as DNP. The two aminonitrophenols did not separate completely. The 4-amino isomer moved rather faster than the 2-amino compound but became diffuse. The result was a broad band which was pink at the bottom and orange at the top, the pink compound being 4-amino-2-nitrophenol. However, by paper-partition chromatography, all three compounds could be separated. The R_F of DNP was 0·60–0·65, for 2-amino-4-nitrophenol it was 0·42–0·44 and for 4-amino-2-nitrophenol it was 0·30–0·32. For the purpose of identifying and isolating the metabolic product of DNP, the two chromatographic methods were combined. An ethanolic extract of liver homogenate after incubation with DNP was added to an alumina column. A yellow band of unchanged DNP appeared and was eluted. This was followed by a pink band. Finally, after elution of the pink band, there remained at the top of the column an orange band which could only be removed after increasing the water content of the developing fluid to about 20% and it was found that this fluid did not contain a diazotizable amine.

The pink fraction obtained from the alumina column produced an orange solution after elution. This solution was evaporated to dryness in an atmosphere of nitrogen and the dry residue dissolved in 2–4 ml. of ethanol. Approximately 20 μl. of this solution was placed upon a paper strip and developed. After completion of the chromatogram three spots were seen: a yellow spot of the same R_F value as DNP; a brownish pink spot of R_F value of 4-amino-2-nitrophenol; and a faint orange spot at the position of 2-amino-4-nitrophenol. Subsequent analysis after cutting the spots from the paper revealed that 90% of the total amine content of the extract was 4-amino-2-nitrophenol and the orange spot accounted for the remaining 10%.

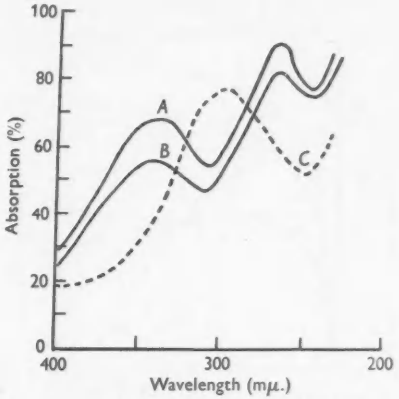

Fig. 4. Ultraviolet absorption of aminonitrophenols compared with metabolite. *A*, 0·0015% 4-amino-2-nitrophenol in 0·001 N-HCl. *B*, metabolite washed from paper chromatogram with 0·001 N-HCl. Estimated to contain 0·0012% of 4-amino-2-nitrophenol. *C*, 0·001% 2-amino-4-nitrophenol in 0·001 N-HCl.

Further evidence of identity was supplied by spectrography. After preliminary separation on an alumina column and concentration into a small volume the amine fraction was placed on a paper strip and developed. The compound of the R_F value of 4-amino-2-nitrophenol was cut from the paper and dissolved in 0·001 N-hydrochloric acid. Its ultraviolet absorption spectrum was observed and compared with that of 4-amino-2-nitrophenol and of 2-amino-4-nitrophenol (Fig. 4). It is seen that the absorption curve of the metabolite of DNP strongly resembles that of 4-amino-2-nitrophenol.

Thus from the evidence of chromatography and spectrography it is concluded that 4-amino-2-nitrophenol is the main reduction product of DNP in liver homogenates.

The relationship between the rate of DNP destruction and the rate of formation of amino compounds was next investigated. An experiment was made in which the amount of DNP destroyed was measured together with the amount of amine produced. The latter, calculated as 4-amino-2-nitrophenol, was determined in the trichloroacetic

acid supernatant and was thus equivalent to the total amine. It was observed that during the early stage of rapid removal of DNP there was a rapid increase in the concentration of amino compounds, but that after 3 hr. loss of amine occurred while DNP destruction continued (Fig. 5).

Earlier in this paper it has been noted that the bulk of the amino compounds were extracted by ether from acidified solutions. However, examination of the residual aqueous solution showed that some amine was still present. This compound was not extractable by ether, benzene or light petroleum, behaving in this respect as 2:4-diaminophenol. This fraction was termed the 'ether-insoluble' fraction. A similar experiment to that described above was performed with the additional elaboration of extracting the trichloroacetic acid solution with ether. The ether-soluble and ether-insoluble fractions were then analysed for amino compounds. Fig. 6 shows that as the DNP is destroyed the ether-soluble amine at first increases rapidly, but later decreases in concentration. At the same time there is a slow increase of the ether-insoluble amine the level of which is maintained after the concentration of the ether-soluble amine begins to fall. The decrease of the ether-soluble fraction commences after 3 hr. incubation. Thus it would appear that 4-amino-2-nitrophenol is itself destroyed.

The destruction of the two aminonitrophenols was next investigated. In turn, each of the above compounds was incubated with liver homogenates. At intervals samples were removed and analysed for ether-soluble amine and ether-insoluble amine. The results of these experiments are shown in Figs. 7 and 8. Both compounds were destroyed by homogenates but at different rates. The 2-amino-4-nitrophenol is slowly destroyed with a gradual accumulation of the ether-insoluble compound. 4-Amino-2-nitrophenol is more rapidly removed. In 5 hr. very little of the ether-soluble 4-amino-2-nitrophenol is left, but there is only a slight increase of the water-soluble compound.

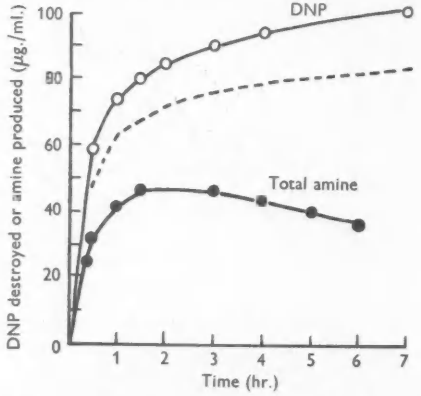

Fig. 5. Comparison between DNP destroyed and amine produced by liver homogenate. Initial DNP concentration 100 μg./ml. homogenate. Dotted line is theoretical amount of 4-amino-2-nitrophenol to be expected calculated from DNP destroyed assuming 100% recovery and no destruction of amine. ○—○, DNP destroyed; ●—●, amine produced.

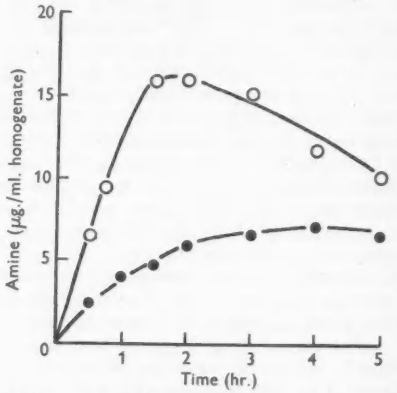

Fig. 6. Comparison between production of ether-soluble and ether-insoluble amine during course of DNP destruction. Initial concentration of DNP, 100 μg./ml. (Trichloroacetic acid supernatant fractionated and analysed as described in text.) Results read as 4-amino-2-nitrophenol. ○—○, ether-soluble amino compounds; ●—●, ether-insoluble amino compounds.

That the mechanism of this destruction was similar to the destruction of DNP and might possibly involve the same enzymes was demonstrated by experiment. It was found that the destruction of the amino nitro compounds could be inhibited by heat and cyanide ions but not by sodium azide, sodium malonate, *p*-chloromercuribenzoic acid or iodoacetic acid. Strict summation experiments were difficult to perform as the product of DNP reduction, 4-amino-2-nitrophenol, could not be distinguished by the method of estimation from the added amine. Moreover, the 4-amino-2-nitrophenol is itself destroyed by the liver homogenate and might also compete for the enzyme system. An indication that the presence of amino-nitrophenols lowers the rate of DNP destruction and may be competing for the enzyme system was obtained by the experiment illustrated in Fig. 9. Three quantities of homogenate were prepared. The first contained DNP, the second DNP and 2-amino-4-nitrophenol and the third DNP and 4-amino-2-nitrophenol.

Samples were taken from each homogenate at intervals of 15 min. It is seen that both compounds inhibit the destruction of DNP, but that 4-amino-2-nitrophenol has the greater effect.

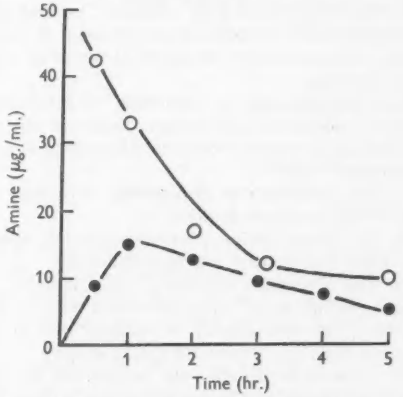

Fig. 7. Rate of destruction of 4-amino-2-nitrophenol in liver homogenate. Initial amine concentration of 100 μg./ml. Trichloroacetic acid supernatant fluid analysed as described in text. ○—○, 4-amino-2-nitrophenol; ●—●, ether-insoluble amine.

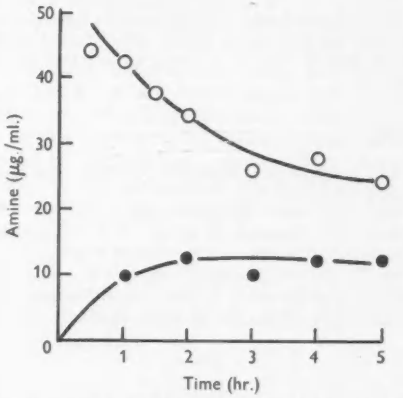

Fig. 8. Rate of destruction of 2-amino-4-nitrophenol in liver homogenate. Initial amine concentration of 100 μg./ml. Trichloroacetic acid supernatant fluid analysed as described in text. ○—○, 2-amino-4-nitrophenol; ●—●, ether-insoluble amine.

It has been shown that DNP is reduced *in vitro* by animal tissues to 4-amino-2-nitrophenol with only a slight amount of 2-amino-4-nitrophenol. It was, therefore, of interest to compare this result with the urine excretion products of rats poisoned by DNP. Male rats of 200 g. weight were given 20 mg. DNP/kg. body weight by subcutaneous injection. The animals were kept in metabolism cages for the next 24 hr. and their urine collected. The urine was extracted in the same way as described for the homogenates. The dried extract was dissolved in a few drops of ethanol. Approximately 20 μl. of the extract was then developed on a paper chromatogram and compared with a known solution containing DNP and the two isomeric aminonitrophenols. Two spots were obtained from the urine extract. One had an R_F value identical with the spot due to pure DNP. The second spot was orange in colour and had the R_F value of 2-amino-4-nitrophenol. There was no sign of 4-amino-2-nitrophenol.

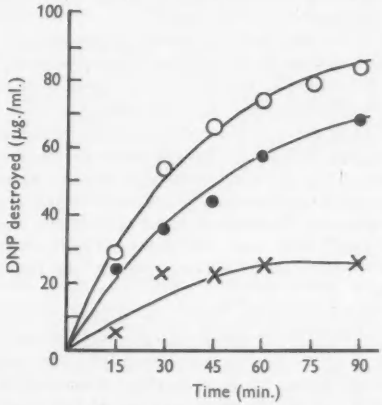

Fig. 9. Influence of aminonitrophenols on rate of destruction of DNP. ○—○, normal homogenate containing DNP at initial concentration of 100 μg./ml.; ●—●, homogenate containing DNP at 100 μg./ml. + 2-amino-4-nitrophenol at 200 μg./ml.; ×—×, homogenate containing DNP at 100 μg./ml. + 4-amino-2-nitrophenol at 200 μg./ml.

It has been shown that the latter compound is more rapidly destroyed *in vitro* than its isomer. It might be expected, therefore, that 2-amino-4-nitrophenol would appear as the predominant reduction product of DNP in the urine.

It has been remarked above that the 2-amino isomer was the compound found in the urine of men and dogs poisoned by DNP (Magne *et al.* 1932). It is of interest to note that the latter workers found 4-amino-2-nitrophenol in the liver of a DNP-poisoned dog.

DISCUSSION

The enzymic reduction of aromatic nitro groups is well known. Westfall (1943) and Bueding & Jolliffe (1946) have demonstrated that trinitrotoluene (TNT) is reduced by the succinic oxidase system to an amino derivative. The nitro group of chloromycetin is reduced by bacterial enzymes (Smith & Worrel, 1949) and has been related to a nitrite

reductase (Egami, Ebata & Sato, 1951). Greville & Stern (1935) showed that a bacterial succinic dehydrogenase would reduce 2:4-dinitrophenol. The present paper confirms that animal tissues can reduce one nitro group of DNP to an amino group. The process is similar to that described for TNT inasmuch that substrates of dehydrogenases, lactate and succinate, increase the rate of reduction. However, it was not possible to reduce DNP by a preparation of heart-muscle succinic oxidase. In this connexion, it may be noted that Bueding & Jolliffe did not obtain a reduction of TNT unless coenzyme I was added. Further work is necessary to see if there is any relation between the reduction of DNP with the respiratory activity of the tissue.

The next stage of the process, destruction of the amine, appears to be similar in properties to the first stage as both are inhibited by cyanide ions and not by the other enzyme inhibitors mentioned. It is possible that the reduction of the nitro group in the 4 position is followed by reduction of the 2-nitro group. Pure 2:4-diaminophenol is not extracted from water by organic solvents and would therefore be contained in what is here termed the 'ether-insoluble' fraction. Definite identification of this fraction as 2:4-diaminophenol has not been made.

It is interesting that the amino compound appearing in the urine of animals poisoned by DNP is the 2-amino-4-nitro compound. The experiments reported above show that this compound is destroyed less rapidly than its isomer. The excretion products in the urine represent the balance of production and destruction of both aminonitrophenols.

SUMMARY

1. 2:4-Dinitrophenol is reduced by rat-liver homogenates to 4-amino-2-nitrophenol. Some 2-amino-4-nitrophenol is obtained (about 10 % of the total amine).

2. Maximum rate of reduction of 2:4-dinitrophenol is attained in phosphate buffer of pH 7 at 37° and in the presence of sodium lactate or sodium succinate.

3. The reaction is completely inhibited by 5×10^{-3} M-potassium cyanide.

4. Rat spleen, kidney, intrascapular fat, muscle and brain also destroy 2:4-dinitrophenol.

5. The reduction products were isolated by means of alumina chromatograms and paper-partition chromatography. 4-Amino-2-nitrophenol was identified by ultraviolet spectrography.

6. Liver homogenates can reduce the isomeric aminonitrophenols. The second reduction product is thought to be 2:4-diaminophenol, but has not been identified.

7. The main excretion products of 2:4-dinitrophenol in the urine of rats were unchanged 2:4-dinitrophenol and 2-amino-4-nitrophenol.

My thanks are due to Dr S. Ellingworth, Messrs Imperial Chemical Industries Ltd., for the gift of the aminonitrophenols and to Miss A. E. Scott for valuable technical assistance.

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The Metabolism of some Saturated Aliphatic Hydrocarbons, Alcohols and Fatty Acids by *Proactinomyces opacus* Jensen (*Nocardia opaca* Waksman & Hendrik)

By D. M. WEBLEY
Macaulay Institute for Soil Research, Aberdeen

and P. C. DE KOCK
Botany Department, The University, Aberdeen

(*Received* 26 *September* 1951)

Erikson (1949), as a result of a study of about 300 strains of Proactinomycetes, mostly isolated from Rothamsted soils, came to the conclusion that the partially acid-fast strains in that locality were mainly accounted for by *Proactinomyces opacus*, *Pro. salmonicolor* and *Pro. paraffinae*. All three species grow well on liquid paraffin or paraffin wax as the sole energy source.

A few papers have been published on the oxidation of aliphatic hydrocarbons by washed suspensions of pure cultures of micro-organisms. Both Johnson, Goodale & Turkevitch (1942) and Imelik (1949) used species of *Pseudomonas*. It was shown by Johnson *et al.* (1942) that aliphatic hydrocarbons ranging from C_6 to C_{12} were oxidized. Saz (1949), using four strains of pathogenic mycobacteria, found that in particular the 14-, 16- and 18-membered hydrocarbons, both saturated and unsaturated, greatly increase the oxygen uptake. Cetyl (hexadecyl) and stearyl (octadecyl) alcohols had a similar effect. A useful review on the assimilation of hydrocarbons by micro-organisms has been published by ZoBell (1950).

In this paper we have made a study of the aerobic metabolism of a strain (T16) of the non-pathogenic *Pro. opacus* (Jensen) (*Nocardia opaca* Waksman & Hendrik, *Bergey's Manual*, 6th ed.), employing the Warburg technique.

METHODS

Growth of the organism. Pro. opacus, strain T16, isolated by Erikson from soil, was used throughout this work.

The organism was grown in liquid culture. Glucose proved to be the most suitable carbon source for maximum yield of cells. Although good growth took place in the presence of *n*-dodecane and *n*-tetradecane, it was found impossible to separate the cells from the hydrocarbons by centrifuging. The final medium employed was of the following composition: glucose, 20 g.; $NaNO_3$, 2 g.; KH_2PO_4, 0·5 g.; NaCl, 0·2 g.; $MgSO_4$, 0·2 g.; $FeCl_3$, trace; 1 l. distilled water; pH adjusted to 7·2. The medium was distributed in 500 ml. media storage bottles (200 ml./bottle) and inoculated with 8 ml. of a 48 hr. culture of the organism. Growth was carried out at room temperature on a rotary shaking machine. After 5–6 days, the cells were harvested by centrifuging, washed once with distilled water and finally suspended in distilled water. 1 ml. of suspension was usually made to contain about 6 mg. (dry wt.) of organism. The suspension could be kept for periods up to 2–3 weeks at 2° until needed. The experiments quoted in this paper have been performed with suspensions stored for 7–14 days at 2°.

Oxygen uptake. This was measured in a Warburg apparatus at 20°, the final total volume in each vessel being 2·5 ml., made up as follows: 1 ml. suspension; 0·5 ml. 0·066M phosphate buffer, pH 7·0; substrate and distilled water.

Owing to the high endogenous respiration, particularly when newly harvested, it was usually necessary to leave the organism and phosphate buffer shaking gently in the vessels overnight. This preliminary incubation, while markedly reducing the blank, had little effect on the oxidation of the subsequently added substrates. Next morning, 0·2 ml. of 5% (w/v) KOH and fluted filter paper, for the absorption of CO_2, were introduced into the centre cup of each vessel. Distilled water was added directly to the vessel and substrates, if liquid, were tipped in from the side arm after equilibration. In the case of some hydrocarbons and alcohols, it was found advantageous to add 0·2 ml. distilled water to the side arm simultaneously to facilitate tipping, so ensuring that all the substrate passed into the main vessel. In the case of solid substances, these were added directly just before the equilibration period of 10–15 min. For the measurement of R.Q., three manometers were used for each determination (Umbreit, Burris & Stauffer, 1948).

RESULTS

Oxygen uptake of washed suspensions in the presence of liquid aliphatic hydrocarbons

Fig. 1 gives the result of an experiment showing the effect on the oxygen uptake of suspensions of *Pro. opacus* of the addition of the hydrocarbons decane, undecane, dodecane, tetradecane and hexadecane. It is evident that the higher paraffins (dodecane, tetradecane and hexadecane) most markedly affect the oxygen uptake. Saz (1949), working with tubercle bacilli, also found that with tetradecane, hexadecane and octadecane the oxygen uptake was

greatly increased. With *Pro. opacus* it was similarly observed (Saz, 1949) that the organism tended to migrate to the hydrocarbon-water interface.

As the organism is frequently cultured on liquid paraffin, it was thought of interest to find the effect of this substance on the rate of oxygen uptake by

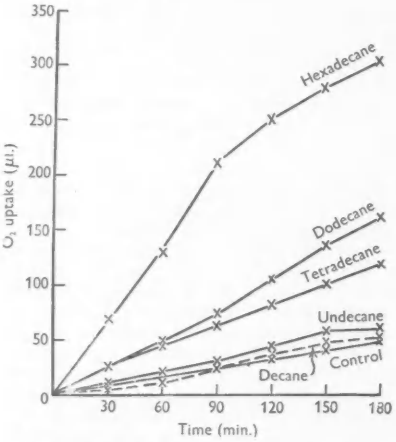

Fig. 1. The oxygen uptake of *Proc. opacus* in the presence of *n*-decane, *n*-undecane, *n*-dodecane, *n*-tetradecane and *n*-hexadecane. A washed suspension (1 ml.) of organism + 0·5 ml. phosphate buffer, pH 7·0, shaken gently overnight. Distilled water and substrates (0·2 ml. from side arm) added next morning. Temp. = 20°.

Pro. opacus. The results are given in Table 1. It appears from Table 1 that liquid paraffin is not as good a substrate as dodecane and tetradecane. Growth experiments also showed that better growth took place on dodecane and tetradecane than on liquid paraffin when these substances were used as sole carbon source in a mineral salt medium of the same composition as given under Methods (the hydrocarbons replacing the glucose).

Table 1. *The oxygen uptake of* Proactinomyces opacus *in the presence of liquid paraffin, dodecane and tetradecane*

(A washed suspension (1 ml.) of *Pro. opacus* + 0·5 ml. of 0·066 M-phosphate buffer, pH 7·0, incubated overnight. Next morning 0·2 ml. hydrocarbon and distilled water added to give a final volume of 2·5 ml.)

Addition	O_2 Uptake in 120 min. (μl.)
None	36
Liquid paraffin	40
n-Dodecane	152
n-Tetradecane	92

Oxygen uptake in the presence of solid hydrocarbons

Solid paraffins increased the oxygen uptake of *Pro. opacus*. Included in Table 2 are dodecane, tetradecane and hexadecane (liquid at 20°) for comparison.

Table 2. *Oxygen uptake of* Proactinomyces opacus *in the presence of some solid aliphatic hydrocarbons*

(A washed suspension (1 ml.) of *Pro. opacus* + 0·5 ml. of 0·066 M-phosphate buffer, pH 7·0, incubated overnight at 20°. Next morning substrates and distilled water were added to give a final volume of 2·5 ml.)

Addition	O_2 uptake in 120 min. (μl.)
None	23
Paraffin wax (0·2 g.)	56
n-Octadecane (0·2 g.)	95
n-Hexadecane (0·2 ml.)	173
n-Tetradecane (0·2 ml.)	82
n-Dodecane (0·2 ml.)	90

The effect of hydrocarbons on oxygen uptake in the presence of glucose

It will be seen from Table 3 that no stimulation of oxygen uptake occurs when glucose and hydrocarbon are present together. In fact, the sum of the separate rates is greater than when the two substrates are oxidized simultaneously. Johnson *et al.* (1942) report similar findings for *Pseudomonas* sp. when glucose and octane were added separately and together to their suspensions. These workers came to the conclusion that 'the fact that they are largely additive indicates that different dehydrogenase systems are involved'.

It is also of interest to note from Table 3 that the rate of oxygen uptake in the presence of glucose is substantially the same as for hexadecane.

Table 3. *The effect of hydrocarbons on the oxygen uptake of* Proactinomyces opacus *in the presence of glucose*

(A washed suspension (1 ml.) of *Pro. opacus* + 0·5 ml. of 0·066 M-phosphate buffer, pH 7·0, shaken overnight at 20°. Next morning substrates and distilled water added to give a final volume of 2·5 ml.)

Addition	O_2 uptake in 120 min. (μl.)
None	27
Glucose (0·4%, w/v)	196
n-Dodecane (0·2 ml.)	102
Glucose (0·4%, w/v) + *n*-dodecane (0·2 ml.)	265
n-Hexadecane (0·2 ml.)	192
Glucose (0·4%, w/v) + *n*-hexadecane (0·2 ml.)	322

Determination of R.Q. *of dodecane*

The theoretical R.Q. for the complete oxidation of a hydrocarbon is 0·65. 'For dodecane, the value obtained was 0·72 and that for the endogenous

respiration was 1·0. Saz (1949) found an identical value for one of his strains (H 37 Ra) using tetradecane. To account for the high value obtained he came to the conclusion that 'if the hydrocarbon is being oxidized, this would suggest that intracellular decarboxylation mechanisms are being stimulated as well'.

Reduction of methylene blue by extract from crushed cells

In order to obtain a sufficient weight of organism for this purpose, the organism was grown on the medium as previously given (see under Methods) but solidified with 2 % agar. The poured plates were inoculated over the whole surface using a sterile glass spreader. The plates were incubated for 14 days at 23°, the surface growth was then harvested, washed once by centrifuging and then deposited in a tared tube by centrifuging. The cells were crushed by McIlwain's method (McIlwain, 1948), using Microid polishing alumina, Grade 3/50, slow cutting (Griffin and Tatlock Ltd.). After centrifuging, the supernatant was used for Thunberg experiments.

Table 4. *Reduction of methylene blue by the extract obtained from crushed cells of* Proactinomyces opacus

(The organisms (4·3 g. wet wt.) were crushed with 11 g. polishing alumina, taken up in 10 ml. phosphate buffer, pH 7·0, and centrifuged. The supernatant liquid was used in hooked Thunberg tubes, each of which contained 1 ml. of supernatant, 0·3 ml. 1/10 000 methylene blue and 0·2 ml. of substrate, etc., to give a total volume of 1·5 ml. Methylene blue and substrate were added from the 'hook' after evacuation and filling with O_2-free N_2.)

Addition	Time after which decoloration obtained (min.)
None	35
Dodecane	17
Hexadecane	17
Decane	40
Sodium lactate (0·2 ml. of 1 %)	20

From Table 4 it will be seen that the rate of methylene-blue reduction is increased in the presence of dodecane and hexadecane. Decane was ineffective. It also had little effect on the oxygen uptake (Fig. 1). Sodium lactate has been included for purposes of comparison.

Metabolism of saturated aliphatic alcohols

Table 5 gives the results of an experiment with ethyl, butyl, amyl, *iso*amyl, *iso*hexyl, heptyl, octyl, decyl, lauryl, cetyl and octadecyl alcohols. It is clearly seen that the long-chain alcohols (decyl, lauryl (dodecyl) and cetyl (hexadecyl)) have a marked effect on the oxygen uptake, while butyl, amyl, *iso*amyl, *iso*hexyl and heptyl alcohols are all toxic and no oxygen uptake is obtained in their presence. Saz (1949) found, too, that the oxygen uptake of the four strains of tubercle bacilli used by him were increased by long-chain (cetyl and stearyl (octadecyl)) alcohols. In the experiments with the immiscible liquid and solid hydrocarbons and alcohols some variation was encountered in the amount of increase in oxygen uptake observed for different experiments. We believe this is connected with the difficulties in introducing the exact amounts (particularly in the case of solid substances) into the main vessel and also to the different degrees of dispersion obtained. The substances in the absence of the cells did not take up oxygen.

Table 5. *Oxygen uptake of* Proactinomyces opacus *in the presence of some saturated monohydric aliphatic alcohols*

(A washed suspension (1 ml.) of *Pro. opacus* + 0·5 ml. 0·066 M-phosphate buffer, pH 7·0, incubated overnight at 20°. Next morning 0·2 ml. of liquid alcohols + 0·2 ml. distilled water added from side arm. In case of solid alcohols 0·2 ml. of the melted alcohol was added directly to suspension. Distilled water added to give final volume of 2·5 ml.)

Addition	O_2 uptake in 120 min. (μl.)
None	15
Ethanol	104
Butanol	Nil
Amyl alcohol	Nil
*iso*Amyl alcohol	Nil
*iso*Hexyl alcohol	Nil
Heptyl alcohol	Nil
Octyl alcohol	16
Decyl alcohol	76
Dodecyl alcohol	98
Hexadecyl alcohol	101
Octadecyl alcohol	47

Metabolism of saturated aliphatic fatty acids

When used in low concentrations (0·0012 M) the longer chain acids give greater oxygen uptakes than acetate, propionate or butyrate (Fig. 2). This agrees with the findings of Randles (1950) for *Neisseria catarrhalis*. From Table 6 it will be seen that the very long-chain saturated fatty acids (laurate, myristate, palmitate, stearate and behenate) behaved in a similar way, although behenate is not so effective as the others.

In higher concentrations (1 %, w/v) caprylate (octanoate), caprate (decanoate), undecanoate and laurate completely inhibited oxygen uptake. The toxic effect of high concentrations of caprylate, caprate, undecanoate and laurate may be connected with their detergent properties.

Effect of fatty acids on oxygen consumption in the presence of glucose

The addition of the long-chain fatty acids (laurate and caprate) and glucose simultaneously to the washed suspension (Table 7) gave analogous results to those obtained for the hydrocarbons (see Table 3).

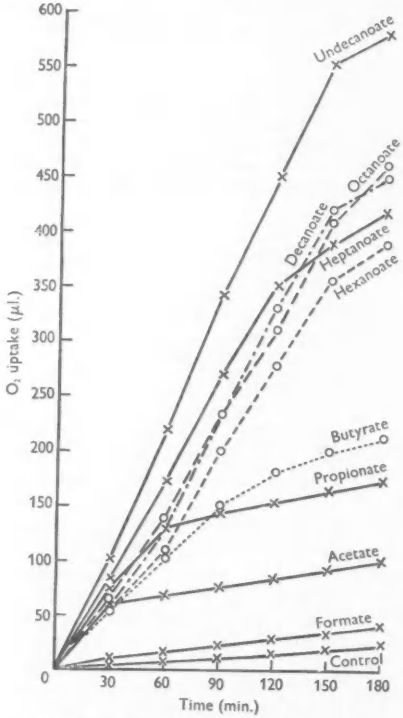

Fig. 2. The O_2 uptake of *Pro. opacus* in the presence of saturated aliphatic fatty acids. A washed suspension (1 ml.) of organism + 0·5 ml. phosphate buffer, pH 7·0, shaken gently overnight at 20°. Next morning substrates (0·5 ml. of 0·06 M from side arm) and distilled water added to give a final concentration of 0·0012 M-fatty acid as sodium salt on total volume of 2·5 ml.

DISCUSSION

Little work has been done on the metabolism of the partially acid-fast proactinomycetes. (For details regarding acid-fastness in this group see Erikson (1949). The capacity of these organisms to grow on liquid paraffin and paraffin wax has been known for a long time (Jensen, 1931). From work reported in this paper it is clear that a wide range of hydrocarbons and closely related compounds increase the rate of oxygen uptake by washed suspensions of *Pro. opacus*. Of the hydrocarbons utilized, the higher members, dodecane, tetradecane and hexadecane were superior to the other members tried (Fig. 1). In this respect *Pro. opacus* differs from the Gram-negative, non acid-fast pseudomonas species as used by Johnson *et al.* (1942), who found that octane was the most actively used substance. Saz (1949), working with pathogenic strains of mycobacteria, obtained similar results to ourselves. He suggested two possibilities to account for the increased oxygen uptake: (*a*) that the hydrocarbons are used as energy source by the organism, and (*b*) that the hydrocarbons alter the respiration rate by changing the total free surface of the cells.

Table 6. *The oxygen uptake of* Proactinomyces opacus *in the presence of long-chain fatty acids*

(A washed suspension (1 ml.) of *Pro. opacus* + 0·5 ml. of 0·066 M-phosphate buffer, pH 7·0, shaken overnight at 20°. Next morning 0·5 ml. of 0·006 M-sodium salt of acid tipped from side arm. Distilled water added to give final concentration of 0·0012 M and final volume of 2·5 ml.)

Addition	O_2 uptake in 120 min. (μl.)
None	17
Laurate (dodecanoate)	204
Myristate (tetradecanoate)	275
Palmitate	214
Stearate	193
Behenate (docosoate)	37

Table 7. *The effect of fatty acids on the oxygen consumption of* Proactinomyces opacus *in the presence of glucose*

(A washed suspension (1 ml.) of *Pro. opacus* + 0·5 ml. of 0·066 M-phosphate buffer, pH 7·0, shaken overnight at 20°. Next morning substrates and distilled water added to give final volume of 2·5 ml.)

Addition	O_2 uptake in 120 min. (μl.)
None	47
Glucose (0·4 %, w/v)	195
Sodium dodecanoate (0·0012 M)	225
Glucose (0·4 %, w/v) + sodium dodecanoate (0·0012 M)	366
Sodium decanoate (0·0012 M)	134
Sodium decanoate (0·0012 M) + glucose (0·4 %, w/v)	242

In the case of *Pro. opacus*, good growth is obtained using dodecane and tetradecane as sole carbon source in the medium. Saz (1949), on the other hand, only obtained stimulation of growth of one of his strains. The other strains tested were apparently unable to utilize the hydrocarbons as energy source.

As to the question of whether the oxygen uptake observed represents a true oxidation of the substrate added, our growth experiments would

support the view that the hydrocarbons are being actively metabolized. This is further borne out by the results obtained on the partially oxidized derivatives, such as long-chain monohydric alcohols and long-chain monocarboxylic acids. Methylene-blue reduction, moreover, points to the presence of a dehydrogenase enzyme present in the cells capable of oxidizing the long-chain hydrocarbons.

It seems that this organism offers very interesting possibilities for further studies on metabolism.

SUMMARY

1. The oxygen uptake of washed suspensions of *Proactinomyces opacus* is increased in the presence of *n*-dodecane, *n*-tetradecane, *n*-hexadecane, *n*-octadecane and paraffin wax.

2. Decyl, lauryl (dodecyl) and octadecyl alcohols give increased oxygen uptake, but amyl, *iso*amyl, *iso*hexyl, and heptyl alcohols are toxic.

3. The long-chain fatty acids (C_7–C_{16}) are all actively metabolized at very low concentrations (0·0012M). At 1%, octanoate, decanoate, undecanoate and laurate (dodecanoate) are toxic.

4. The rate of methylene-blue reduction by the supernatant liquid obtained from the crushed cells of *Proactinomyces opacus* is increased in the presence of dodecane and hexadecane.

We are grateful to Mrs D. Oxford for suggesting this problem and to her and to Dr G. K. Fraser for their continued interest in this work. The work was carried out in the Botany Department, and we are grateful to Prof. J. R. Matthews for the facilities provided. We also wish to thank Messrs Hess Products Ltd., Littleborough, for gifts of fatty acids and to others for gifts of hydrocarbons of high purity.

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Reduction of Dehydroascorbic Acid by Bacteria

2. ROLE OF CYTOCHROME IN HYDROGEN TRANSPORT

By B. P. EDDY, M. INGRAM and L. W. MAPSON
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research

(*Received* 24 *August* 1951)

In a previous paper (Eddy, 1952) it was shown that the reduction of dehydroascorbic acid (DHA) to ascorbic acid (AA) by *Escherichia coli* can be effected with hydrogen supplied by various hydrogen donator/dehydrogenase systems in intact cells, but that corresponding cell-free systems are ineffective. It was therefore suggested that some additional factor, possibly a hydrogen carrier, is necessary.

Our attention was first directed to cytochrome because it was reported that some fifteen strains of streptococci were unable to reduce DHA (Gunsalus & Hand, 1941) and we found the same with species of *Lactobacillus* and *Clostridium*. These bacteria can produce reducing conditions of sufficiently high intensity for the reduction of DHA to be thermodynamically possible, but possess no cytochrome (Fujita & Kodama, 1934; Frei, Riedmüller & Almasy, 1934). When a connexion between DHA reduction and cytochrome was in fact found in *Esch. coli*, comparison of the two properties was naturally extended over a range of bacterial types.

The present paper falls, therefore, into two parts, the first describing attempts to relate DHA reduction to the hydrogen carrier and the second being a survey of the types of bacteria able to effect the reduction.

METHODS

For spectroscopic work the organisms were grown in bulk on a solid medium containing 1% Protex, 1% peptone, 1% gelatin, 0·5% Yeastrel, 0·5% glucose, 1% $CaCO_3$ and 3% agar. After harvesting, the cells were washed twice with Ringer's solution (quarter strength) and suspended at the required concentration either in this solution or in phosphate buffer at pH 6·2.

Organisms to be tested for DHA reduction were grown in nutrient broth + 0·5 % glucose in 1 oz. screw-capped bottles in which they were centrifuged, washed once with Ringer's solution and finally suspended in buffer. Their ability to reduce DHA was tested as described previously (Eddy, 1952) using 0·02 M-glucose as hydrogen donator.

For viewing cytochrome spectra a Zeiss microspectroscope was used. Investigations at liquid air temperature were carried out as described by Keilin & Hartree (1949). For estimating changes in the intensities of cytochrome bands the microspectroscope was fixed vertically above one limb of a Duboscq colorimeter from which the prisms and eyepiece had been removed. A standard suspension of *Esch. coli* ($1·5 \times 10^{11}$ cells/ml.), with added dithionite, was placed in the colorimeter cup and the vernier scale set at 10 mm. The experimental suspension of equal density but without dithionite was placed in a cell of optical depth 10 mm., flooded with liquid paraffin and illuminated horizontally so that both spectra could be viewed simultaneously. The intensities of the spectra were then matched by altering the intensities of their respective light sources. After the addition of DHA the intensities of the spectra and of the cytochrome b_1 bands were rematched, altering the comparison spectrum by varying the intensity of the light source and the depth of suspension interposed.

RESULTS

A. *Connexion between cytochrome and ability to reduce DHA in* Escherichia coli

It seemed that, if cytochrome were needed to reduce DHA, the failure of enzyme preparations to effect the reduction might be due to the absence of cytochrome. Non-reducing cell-free dehydrogenase preparations of *Esch. coli* prepared by tryptic digestion and acetone treatment as described in a previous paper (Eddy, 1952), or mechanically in a Mickle tissue disintegrator, were examined spectroscopically after the addition of sodium dithionite. It was found that in all cases the cytochrome bands were either absent or were so weakened as to be barely visible; this supported the idea that reduction might be connected with cytochrome.

Unfortunately we have not been able to obtain cell-free preparations of the cytochrome from *Esch. coli*, with which to try to restore the reducing capacity of cell-free dehydrogenase preparations, and so we have had to use spectroscopic methods to detect any reaction between DHA and the cytochrome. The experiments have consisted essentially in generating the reduced cytochrome spectrum (b_1 band) by incubating a suspension of *Esch. coli* anaerobically, and then adding DHA (also anaerobically because the band disappears completely on aeration), and seeking to observe a weakening of the absorption band due to oxidation of the cytochrome by the DHA.

The first observations were made visually. They indicated that the intensity of the b_1 band was decreased on adding DHA, but only slightly. Because it was difficult visually to be certain of the oxidation of the band, when it was so incomplete, attempts were made to confirm the observations photographically, using the following technique.

A suspension of *Esch. coli* containing approximately $1·5 \times 10^{11}$ cells/ml. was prepared in phosphate buffer at pH 6·2. 2·5 ml. of this suspension were transferred to a glass cell of optical depth 5 mm. and the surface was flooded with liquid paraffin to maintain anaerobic conditions in the system. 0·1 ml. 0·0625 M-sodium formate was added (at the final concentration the dehydrogenase activity would be about half maximal), the cytochrome allowed to become fully reduced and the spectrum photographed. Urethane (0·1 ml. 50 %, w/v), DHA (0·1 ml. 1 % (w/v); to give maximal rate of reduction with respect to DHA concentration) and finally sodium dithionite, were added in turn and the spectrum photographed after each addition. Both the urethane and DHA solutions were previously de-aerated by evacuation and held under liquid paraffin until required. A definite weakening of the cytochrome b_1 band was recorded photographically after the addition of DHA in such experiments.

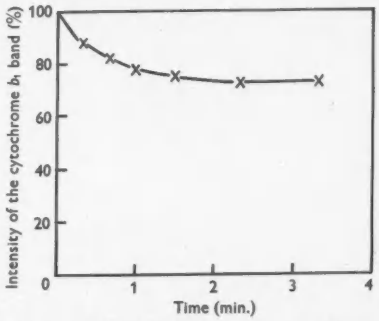

Fig. 1. Effect of dehydroascorbic acid on the fully reduced cytochrome b_1 band in *Esch. coli*. 1 mg. DHA was added to 2·5 ml. suspension containing $1·5 \times 11^{11}$ cells/ml.

Attempts were then made to measure the degree of oxidation of the cytochrome b_1 band, by comparing it in the microspectroscope with that of a standard suspension of *Esch. coli* in which the cytochrome had been completely reduced with dithionite. Using a suspension in which the bands had become fully reduced without added hydrogen donator, and to which urethane had then been added, it was estimated that the addition of DHA resulted in approximately 30 % diminution in the intensity of the cytochrome b_1 band in 3 min. (Fig. 1), indicating 30 % oxidation of the reduced cytochrome. When the urethane was omitted, and sufficient glucose included to reduce all the added DHA, the cytochrome (b_1 band) changed from 70 to

90% reduced in 2·5 hr. and could then be re-oxidized to the same extent as previously by the addition of more DHA.

These observations appeared to reveal a connexion between the cytochrome of *Esch. coli* and its ability to reduce DHA. It was soon evident, however, that some organisms possessing cytochrome were unable to effect this reduction: e.g. baker's yeast had been reported to be ineffective by Gunsalus & Hand (1941) and we confirmed this observation several times. It therefore seemed necessary to compare the ability to reduce DHA with the cytochrome complement in a representative range of bacterial types.

B. *A survey of DHA-reducing ability and cytochrome complement in different genera*

Some information was already available. The cytochrome absorption spectra for a variety of bacteria had been listed by Fujita & Kodama (1934) and by Frei *et al.* (1934). These lists were a guide in choosing types for test, though we always checked the quoted complement against that actually observed in the strains we used. Some information had also been published about the types of organisms able to reduce DHA, and as Stewart & Sharp (1945) had reported that various staphylococci and coliform bacteria are best able to bring about this reduction, we began by testing related organisms.

Representative species of *Eberthella*, *Erwinia*, *Escherichia*, *Proteus*, *Salmonella* and *Serratia* were found to reduce DHA. Gunsalus & Hand (1941) reported that several strains of *Aerobacter cloacae* could also effect the reduction, though a British strain and two strains of *A. aerogenes* tested by us were found to be ineffective. Strains of three species of *Staphylococcus* were also found to be able to reduce DHA. The nomenclature in this paper follows *Bergey's Manual of Determinative Bacteriology*, 5th ed. (1939).

Species from most of the above genera had been examined by Fujita & Kodama (1934) and found to exhibit the cytochrome b_1 band in place of the *b* and *c* bands of other types. Hence a series of types characterized by other cytochrome complements was next examined—species of *Clostridium* (1), *Lactobacillus* (1), *Acetobacter* (2), *Pseudomonas* (1), *Bacillus* (8), *Micrococcus* (7) and *Sarcina* (1)—and all were found to be unable to reduce DHA.

These observations suggested a correlation between ability to reduce DHA and the possession of cytochrome b_1, and our belief in this connexion was strengthened on careful examination of several apparently irregular cases:

(1) It had been reported (Tkachenko, 1936) that DHA was reduced by *Lactobacillus acidophilus*. As species of this genus possess no cytochrome, we carefully examined three strains of this species, and found no evidence of reducing power. Reference to the technique employed by Tkachenko (1936) has led us to regard his evidence as inconclusive.

(2) On examination of a strain designated *Staphylococcus citreus* which failed to reduce DHA, it was found to possess a spectrum with well defined cytochrome *b* and *c* bands, and further systematic examination revealed that it was, in fact, *M. conglomeratus*. It thus fell into line with the other *Micrococcus* species examined, namely *M. aurantiacus*, *M. candicans*, *M. flavus*, *M. freudenreichii*, *M. luteus*, *M. lyso-deikticus* and *M. ureae*.

(3) Among the Gram-negative bacilli related to the coliforms and included in the family Enterobacteriaceae according to *Bergey's Manual*, 5th ed. (1939), Fujita & Kodama (1934) described one species (*Bacillus prodigiosus* syn. *Serratia marcescens*) as possessing cytochromes *b* and *c*. As we found species of *Serratia* able to reduce DHA, we examined their spectra and found that they possessed only a single band, and that the band remained single even on cooling in liquid air, a treatment which sharpens the bands and makes it possible to distinguish readily cytochromes *b* and *c* (Keilin & Hartree, 1949).

(4) The situation in the genus *Bacillus* was particularly interesting. Our examination of strains of eight species (*B. cereus*, *B. licheniformis*, *B. megatherium*, *B. mesentericus*, *B. mycoides*, *B. polymyxa*, *B. pumilus* and *B. subtilis*) showed that none could reduce DHA though, on the other hand, Keilin (1934) had earlier reported that *B. megatherium* possessed a cytochrome b_1 band similar to that in *Esch. coli* and similar reports had been made in respect of *B. subtilis* (Frei *et al.* 1934). Further, Chaix & Roncoli (1951) have recently observed that young cultures of several *Bacillus* species exhibit a single broad absorption band at 550–560 mμ. which they provisionally identify as that of cytochrome b_1. Examination of the spectra of our *Bacillus* strains (in the state in which we tested them for DHA reduction) showed that they mostly possessed single cytochrome bands similar to those described by Chaix & Roncoli. At first, these observations weakened our belief in the connexion between DHA reduction and cytochrome b_1. However, careful examination showed that the cytochrome bands in these *Bacillus* strains were broader than the typical b_1 bands of staphylococci and the Enterobacteriaceae. On heating a suspension of *B. pumilus* to 80° for 20 min. in the presence of dithionite, the long-wavelength side of the band disappeared, leaving a narrow band in the position of the typical *c* band. Again, examination of the 'b_1' band in *B. cereus* at the temperature of liquid air showed a band centred at 552 mμ. with faint shading on either side (possibly indicative of

two additional bands analogous to the spectrum found in old cultures of *B. subtilis* which contains cytochromes *b* and *c*), whereas under these conditions the coliforms show a single band from 553 to 560 mμ. with a barely perceptible maximum at 557 mμ. These observations lead us to conclude that the single band in *Bacillus* species is probably not identical with the classical b_1 band of the coliform organisms, which conclusion agrees with the inability of the *Bacillus* strains to reduce DHA.

It seems, therefore, that ability to reduce DHA is confined to some strains within the genus *Staphylococcus* and the family Enterobacteriaceae, which contain typical cytochrome b_1.

Variation in DHA-reducing capacity among strains. It has become certain, however, that a considerable proportion of organisms apparently containing a perfectly normal cytochrome b_1 are unable to reduce DHA. On further investigation of the two groups of reducing organisms mentioned above, it was found that some members of each were unable to reduce DHA. Six strains each of *Staph. albus* and *Staph. aureus* were provided by Dr R. M. Fry (Public Health Laboratory, Cambridge) and two strains of *Staph. citreus* (7415, 7990) were obtained from the National Collection of Type Cultures (N.C.T.C.). Of these, one strain of *Staph. albus* failed to reduce DHA. Table 1 gives the occurrence of DHA-reducing ability in the strains of species from the Enterobacteriaceae which we have examined; about half are inactive. Similar results had in fact been recorded previously. Gunsalus & Hand (1941) and Stewart & Sharp (1945) stated that, of the strains of coliform organisms they tested, about half did not reduce DHA.

With a strain of *Esch. coli* (N.C.T.C. 7277) which is unable to reduce DHA, experiments similar to those in section *A* did not reveal any alteration in the intensity of the cytochrome band on addition of DHA. Attempts to make similar observations with strains of *Staphylococcus* were indecisive because the cytochrome bands were too weak.

DISCUSSION

The results in the first half of the preceding section, suggesting on spectroscopic evidence that cytochrome b_1 is oxidized by DHA, support, and are supported by, those in the second half, which indicate that a grouping of bacteria according to their complement of cytochromes will delimit a group of genera within which the ability to reduce DHA is confined. Taken together, the observations show that DHA can be reduced by hydrogen transported by cytochrome b_1 but not by other cytochromes.

These relations, be it noted, are those which obtain when dehydrogenases of the anaerobic type effect the reduction of DHA. With other hydrogen-donating systems different relations may hold: for example, glycine can act as a hydrogen donator for DHA reduction (Eddy, 1952, Table 1), but the dehydrogenase probably involved is an autoxidizable flavoprotein. Such a system might not be expected to show any relation to cytochrome, but the fact that cytochrome is not an essential inter-

Table 1. *Ability to reduce dehydroascorbic acid among members of the family Enterobacteriaceae*

(Numbers quoted are those of N.C.T.C. strains; other strains were supplied by Dr W. J. Dowson, Botany School, Cambridge.)

Species	Reducing strains	Non-reducing strains
Aerobacter aerogenes	—	(2 unknown)
A. cloacae	(U.S. strains)	(1 unknown)
Eberthella belfastiensis (*Bact. coli anaerogenes*)	4450	4174
Erwinia aroideae	(1 unknown)	—
Erwinia carotovora	(4 unknown)	—
Escherichia coli (*Bact. coli* Type I)	8196, (1 unknown)	8179, 8333
Esch. coli (*Bact. coli* Type II)	8164	—
Esch. coli (irregular)	—	7275, 7277
Esch. freundii (*Bact. coli* Inter. I)	—	8165, 8166
Esch. freundii (*Bact. coli* Inter. II)	6072	6071
Klebsiella ozaenae	—	5053
K. pneumoniae	—	2794, (1 unknown)
K. pneumoniae A	—	5054
K. pneumoniae B	—	5055
K. pneumoniae C	—	5056
K. rhinoscleromatis	—	5047
Proteus vulgaris	(1 unknown)	—
Salmonella pullorum	(1 unknown)	—
Serratia marcescens	(1 unknown)	(1 unknown)
Serratia kielensis	4619	—

mediate does not exclude the possibility that the dehydrogenase is capable of reducing it.

It is not known why the cytochrome b_1 band is not fully removed by DHA. It may be either that the residual level of dehydrogenase activity in our experiments was sufficient to keep the cytochrome 70% reduced or, alternatively, that the oxidation-reduction potential of the AA/DHA system, as constituted in this case, was the same as that of 70% reduced cytochrome b_1.

The inability of many organisms containing cytochrome b_1 to effect the reduction raises a further problem. As there is no evidence that this is due either to their cytochromes or cytochrome-reducing mechanisms, we suppose it to be due to lack of a mechanism linking oxidation of the cytochrome with reduction of DHA. In the previous paper (Eddy, 1952) theoretical considerations were given in favour of an enzyme activating DHA so that it can be reduced. The failure of some coliform bacteria to reduce DHA lends weight to these suggestions, and preliminary experiments have shown that the oxidation of leuco-Nile blue by DHA varies both in rate and magnitude, according to whether DHA-reducing or DHA-non-reducing strains of bacteria are present.

SUMMARY

1. In cell-free dehydrogenase preparations from *Escherichia coli*, which are unable to reduce dehydroascorbic acid (DHA), the cytochromes are either absent or greatly decreased in amount.

2. When DHA is added anaerobically to a suspension of *Escherichia coli* the intensity of the fully reduced cytochrome b_1 band becomes diminished by approximately 30%.

3. Amongst organisms examined so far, ability to reduce DHA is confined to members of the genus *Staphylococcus* and the family Enterobacteriaceae, these being the groups in which the *b* and *c* cytochrome bands are replaced by the b_1 band.

4. Approximately half the members of the Enterobacteriaceae tested failed to reduce DHA, and it is suggested that a DHA-activating enzyme may be necessary in addition to cytochrome b_1.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research. The authors wish to express their gratitude to Dr E. F. Hartree and Dr T. Mann, F.R.S., for help and advice received from them.

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Studies in Immunochemistry

11. THE ACTION OF DILUTE ALKALI ON THE *N*-ACETYLHEXOSAMINES AND THE SPECIFIC BLOOD-GROUP MUCOIDS

By D. AMINOFF, W. T. J. MORGAN and WINIFRED M. WATKINS

The Lister Institute of Preventive Medicine, London, S.W. 1

(*Received* 21 *September* 1951)

The increasing use within recent years of the colorimetric method introduced by Morgan & Elson (1934) for the detection and quantitative determination of the *N*-acetylhexosamines calls for a more detailed study of the conditions necessary to obtain reliable and reproducible results and demands a more thorough understanding of the structural changes involved before the results can be applied to complex structures containing *N*-acetylhexosamine components, such as the blood-group mucoids.

Ehrlich (1901) observed that mucins and mucoid substances give rise to an intense purple colour after heating with dilute alkali for a short time and the addition of an acid solution of *p*-dimethylaminobenzaldehyde. If the alkali treatment is

omitted no colour formation takes place. At about the same time Müller (1901) found that pentaacetylglucosamine after heating with dilute potassium hydroxide gave a similar colour on the addition of Ehrlich's *p*-dimethylaminobenzaldehyde reagent, and Zuckerkandl & Messiner-Klebermass (1931) subsequently suggested that a pyrroline derivative was formed from *N*-acetylglucosamine by the action of alkali, and that this substance reacted with Ehrlich's reagent to yield a coloured complex. Morgan & Elson (1934) and Morgan (1938) considered that the formation of a pyrrol derivative was difficult to reconcile with the fact that *N*-trimethylacetyl-, *N*-*o*-bromobenzoyl- and *N*-benzoyl-glucosamines gave a similar colour after treatment with dilute alkali and Ehrlich's reagent and it was suggested that *N*-acetylglucosamine formed an oxazole or oxazoline derivative under these conditions. Some oxazole preparations, made by condensing amides with α-halogenoketones (Blumlein, 1884; Lewy, 1887) gave, without previous alkali treatment, a strong colour with the *p*-dimethylaminobenzaldehyde reagent, and the production of an immediate colour by these substances seemed to support the suggestion that *N*-acetylglucosamine was converted to a heterocyclic structure of this kind. It has been known for some time, however, that the positive colour reaction given by the oxazole preparations is due to the presence of an impurity of unknown nature in the materials examined and that if the oxazole is purified by precipitation as a complex with mercuric chloride (Cornforth & Cornforth, 1947), it fails to give a positive colour reaction with *p*-dimethylaminobenzaldehyde.

A number of authentic oxazoline derivatives were found by Morgan (unpublished observations) to yield no colour with the *p*-dimethylaminobenzaldehyde reagent, but nevertheless White (1940), on the basis of the results of methylation experiments, concluded that the formation of a glucoxazoline (2-methyl-4:5-glucopyrano-Δ^2-oxazoline) occurred when *N*-acetylglucosamine was treated with alkali. Morgan (1938) pointed out that two molecules of *N*-acetylglucosamine might condense together and form a diglucopyrazine structure similar to that proposed earlier by Stolte (1908), who claimed to have obtained ditetrahydroxybutylpyrazine from glucosamine.

In an attempt to understand more fully the structural changes which occur when the *N*-acetylhexosamines are treated with dilute alkali under controlled conditions, such as those employed for their colorimetric determination, a study has been made of the ultraviolet absorption spectra given by these substances after treatment with dilute sodium carbonate or with different buffer systems at known pH values, and of the light absorption of the systems after the development of the purple colour following the addition of the *p*-dimethylaminobenzaldehyde reagent. A number of purified human blood-group substances which are mucoids and are known to behave with alkali and Ehrlich's reagent as do the *N*-acetylhexosamines, have been examined in the same manner. The isolation and identification of the substance which arises from the simple *N*-acetylhexosamines on treatment with alkali and which is responsible for giving the colour with Ehrlich's reagent is being undertaken in collaboration with Dr J. W. Cornforth.

EXPERIMENTAL

Materials and methods

N-Acetylglucosamine. A standard solution of *N*-acetylglucosamine (1%) was kept at 2° and from this, suitable dilutions were made immediately before use.

N-Acetylchondrosamine. The substance was prepared by the acetylation of chondrosamine hydrochloride with acetic anhydride in the presence of silver acetate. (Found: C, 42·9; H, 6·7; N, 6·3; $C_8H_{15}O_6N$ requires C, 43·4; H, 6·8; N, 6·3%.) $[\alpha]_{5461}$ +131°→98° (*c*, 0·5 in water); m.p. 162–164° (uncorr.). The material was found to be chromatographically homogeneous, a single component being observed when a solution of the substance was run in collidine, butanol, butanol-acetic acid, butanol-ethanol or in phenol according to the standard techniques used in paper chromatography. The R_F value of the material in these solvents is very similar to that of *N*-acetylglucosamine run simultaneously. After hydrolysis with 0·5N-HCl for 16 hr. at 100°, the material gave the full hexosamine colour corresponding to an equivalent weight of glucosamine hydrochloride and chromatographic analysis of the hydrolysate revealed the presence of one component only, which had all the properties of chondrosamine hydrochloride. A 1% solution of *N*-acetylchondrosamine was kept at 2° and the appropriate dilutions made immediately before the experiment.

p-Dimethylaminobenzaldehyde reagent (DMAB). A.R. grade *p*-dimethylaminobenzaldehyde was further purified by the method of Adams & Coleman (1944) and finally fractionated by the addition of water to an ethanol solution of the material. The crystals separating from solution within the range 50–75% water are almost colourless and are suitable for use. The reagent was prepared by dissolving 2 g. of the material in 100 ml. of A.R. glacial acetic acid which contains 2·5% (v/v) A.R. 10N-HCl. The solution, which possessed a very pale greenish yellow colour, was stored at 0° in small glass-stoppered bottles. A fresh bottle was opened for each experiment. The use of material which has been repeatedly thawed and frozen or allowed to stand at room temperature should be avoided.

Reducing sugars. Determined according to Somogyi (1937).

Fucose. Determined by the method of Dische & Shettles (1948).

Glucosamine and chondrosamine. Determined as described by Elson & Morgan (1933) using hexosamine concentrations of 10–40 μg. as standards, a photoelectric colorimeter and a green filter (max. transmission 550 mμ.), or a Hilger Uvispek spectrophotometer.

Buffer solutions. As described by Vogel (1939).

A *substance.* The material was obtained from a pseudomucinous cyst fluid (no. 122) according to the isolation procedures described by Aminoff, Morgan & Watkins (1950). $[\alpha]_{5461}$ +11°. N, 5·5% (Kjeldahl). Total fucose, 18·3%. Reducing power, expressed as glucose after acid hydrolysis, 54%. Hexosamine, as base, 32%.

B *substance.* The material was prepared from an ovarian cyst fluid by Mr R. A. Gibbons. The substance showed general chemical properties similar to those recorded for group A substance. N, 5·8%. Total fucose, 18%. Reducing power, expressed as glucose after hydrolysis, 50%. Hexosamine, as base, 20%.

H *substance.* Prepared according to Morgan & Waddell (1945) and Annison & Morgan (unpublished) from cyst fluids obtained from 'secretors' belonging to group O. The material was found to be similar in general chemical properties to the groups A and B substances. N, 5·3%. Total fucose, 14%. Reducing power, expressed as glucose after hydrolysis, 54%. Hexosamine, as base, 31%. $[\alpha]_{5461}$ −30° (*c*, 0·5 in water).

'*Lewis*' Le^a *substance.* Prepared as described by Annison & Morgan (1951) from a cyst fluid obtained from a 'Lewis-positive' patient. The substance, $[\alpha]_{5461}$ −41°, contained N, 5·4%, total fucose, 13% and gave, after hydrolysis with 0·5 N-HCl at 100°, 57% reducing sugars (as glucose) and 32% hexosamine (as base).

Optimal conditions for the colorimetric determination of the N*-acetylhexosamines*

A re-examination of the general procedure used by Morgan & Elson (1934) for the estimation of the *N*-acetylhexosamines was first undertaken.

Period of heating with alkali. *N*-Acetylglucosamine (20 μg.) was measured into each of several test tubes (20 × 1 cm.) graduated at 10 ml. capacity which were carefully selected to possess walls of equal thickness. The sides of the tubes were washed down with water to yield a total volume of 1 ml., and 0·1 ml. of 0·5 N-Na_2CO_3 was added and the contents thoroughly mixed. Each tube was closed by a glass ampoule, the sealed neck of which was inserted into the tube. The ampoules contained 2–3 ml. of water and acted as condensers which prevented loss by evaporation during the short heating periods studied. The tubes were heated up to the level of the liquid contents in a vigorously boiling water bath, and at pre-arranged times up to a maximum period of 15 min., pairs of tubes were withdrawn from the bath and immediately cooled in water at 0° and kept there until all tubes had been heated and cooled. Glacial acetic acid (A.R.) was then run into each tube from a separating funnel to yield a total volume of about 7 ml. and the DMAB reagent (1 ml.) was added. The volume of liquid in each tube was made up to 10 ml. by the addition of glacial acetic acid, was thoroughly mixed and the whole series of tubes allowed to stand at 20° in the dark. A tube containing all the reagents other than the *N*-acetylhexosamine was included to serve as a reagent blank in each experiment. The colour intensity of each solution reached a maximum value after standing for 1·5 hr. at 20°. For routine purposes the intensity of the colour developed is conveniently measured in a simple photoelectric colorimeter using a green filter (max. transmission 550 mμ.) and optical cells 2 cm. deep. In our experience it is advisable to mix the contents of each tube again a few minutes before they are examined in the colorimeter or spectrophotometer.

The relationship between the time of heating with carbonate solution and the intensity of the colour subsequently produced with a fixed amount of DMAB reagent and expressed directly in terms of the galvanometer readings is given in Fig. 1. Under the conditions described the maximum colour was given by a solution which had been heated for 4 min. and it is evident that after this time the structure which gives rise to the coloured complex with the DMAB reagent undergoes fairly rapid destruction, and it is therefore important that the period of heating with alkali should be accurately controlled. The length of the heating period which gives the optimal colour production on the addition of the DMAB reagent varies somewhat according to the thickness and shape of the glass tubes selected, the heating conditions etc., and must be determined for each outfit.

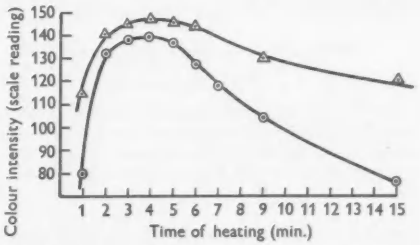

Fig. 1. The influence of the time of heating with Na_2CO_3 on the intensity of the colour produced on the addition of DMAB reagent. ⊙—⊙, *N*-acetylglucosamine; △—△, *N*-acetylchondrosamine.

A similar curve (Fig. 1) was obtained for *N*-acetylchondrosamine, but it was found that nearly five times as much of this amino sugar was required to give a solution of equal colour intensity. Several preparations of *N*-acetylchondrosamine gave similar results. The *N*-acetylchondrosamine chromogen which gives rise to the coloured DMAB complex is apparently less rapidly destroyed than is that derived from *N*-acetylglucosamine. The 4 min. heating period was optimal for quantities from 10 to 50 μg. of the two *N*-acetylhexosamines treated as described above.

Concentration of alkali. The influence of the concentration of Na_2CO_3 and of the period of heating on the amount of colour subsequently developed by *N*-acetylglucosamine after the addition of the DMAB reagent was next examined. Amounts (20 μg.) of *N*-acetylglucosamine were placed in a series of tubes with a solution of Na_2CO_3 of known strength and the total volume of the solution made up to 1·1 ml. with water. The tubes were then heated in a boiling-water bath for predetermined periods, removed in pairs, cooled and treated with glacial acetic acid and DMAB reagent. Duplicate tubes containing 10, 20 and 30 μg. of *N*-acetylglucosamine were likewise heated for 4 min. to act as standards and the maximum colour intensity reached by each pair of tubes, expressed in terms of that given by the *N*-acetylglucosamine standard, was plotted against the time of heating with alkali. The results obtained when three series of tubes containing 0·1, 0·4 and 0·8 ml. of 0·125 N-Na_2CO_3 respectively were examined are given in Fig. 2. *N*-Acetyl-

chondrosamine (100 μg.) was treated in the same manner and the results, expressed in terms of the colour given by *N*-acetylchondrosamine heated for 4 min., are given in Fig. 3. It appears that under these conditions *N*-acetylchondrosamine requires a slightly shorter period of heating with alkali to yield subsequently its maximum colour than does *N*-acetylglucosamine.

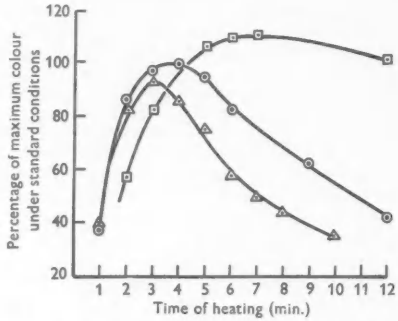

Fig. 2. Effect of the concentration of Na_2CO_3 on the rate and intensity of the colour produced from *N*-acetylglucosamine on the addition of DMAB reagent. ⊡—⊡, 0·1 ml. 0·125 N-Na_2CO_3; ⊙—⊙, 0·4 ml. 0·125 N-Na_2CO_3; △—△, 0·8 ml. 0·125 N-Na_2CO_3.

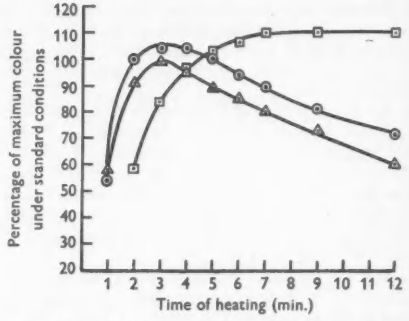

Fig. 3. Effect of the concentration of Na_2CO_3 on the rate and intensity of the colour produced from *N*-acetylchondrosamine on the addition of DMAB reagent. ⊡—⊡, 0·1 ml. 0·125 N-Na_2CO_3; ⊙—⊙, 0·4 ml. 0·125 N-Na_2CO_3; △—△, 0·8 ml. 0·125 N-Na_2CO_3.

Conditions for the standard test. The results of these experiments allowed a set of conditions, the so-called 'standard' test conditions, for the estimation of the *N*-acetylhexosamines to be established. Thus, to a neutral solution of the test material contained in a total volume of 1·0 ml., 0·10 ml. of 0·5 N-Na_2CO_3 is added and the solution is heated for 4 min. in a vigorously boiling water bath, cooled and treated as described above. Standard solutions each containing 10, 20 and 30 μg. of *N*-acetylglucosamine or 50, 100 and 150 μg. *N*-acetylchondrosamine, according to the aminohexose being examined, are included in each determination made. Under the conditions described the pH of the reactants during the heating period is about 10·8.

Influence of different buffer systems and pH *values on the rate and intensity of colour development.* The effect of heating *N*-acetylglucosamine with different buffer systems at the same pH (10·8), and with the same buffers at different pH values, on the maximum amount of colour finally obtained was investigated, using 0·2 ml. of buffer solution in place of the 0·1 ml. of 0·5 N-Na_2CO_3 used in the 'standard' test. Where the heating period necessary to yield the maximum colour exceeded 30 min. the heating was carried out in sealed glass ampoules which contained a total volume of approximately 1·3 ml. and, subsequently, amounts (1·1 ml.) of the cooled reaction mixture which contained exactly 20 μg. of *N*-acetylglucosamine were pipetted into calibrated test tubes, glacial acetic acid and the DMAB reagent added, and the colours allowed to develop in the usual way. The results obtained are given in Table 1. It was observed many years ago that the use of borate buffer at about pH 10·8 in place of Na_2CO_3 at the same pH gave rise to considerably more colour, and the results set out in Table 1 confirm this early observation. The amount of colour obtained on addition of DMAB reagent to *N*-acetylglucosamine heated with glycine buffer at pH 10·8 was almost identical with that obtained with Na_2CO_3.

The experiments were repeated using *N*-acetylchondrosamine, and the results (Table 1) revealed at once that the two amino sugars behaved qualitatively in a similar manner

Table 1. *Amount of colour given by the* N*-acetylhexosamines after treatment at* 100° *with different buffer solutions and the addition of DMAB reagent*

		N-Acetylglucosamine		*N*-Acetylchondrosamine	
Alkaline system	pH	Period of heating for maximum colour production (min.)	Amount of colour expressed as % of amount given by *N*-acetylglucosamine under standard conditions	Period of heating for maximum colour production (min.)	Amount of colour expressed as % of amount given by *N*-acetylglucosamine under standard conditions
'Standard' procedure	10·8	4	100	3–4	23
Potassium borate + KOH	9·0	45	152	50	40
	10·0	12	155	15	35
	11·0	7	156	10	30
Glycine buffer	10·0	110	97	90	25
	10·9	40	100	40	25
	12·2	2	77	1–2	12

with the different buffer systems but that in each instance *N*-acetylchondrosamine gave rise to a maximum amount of colour equivalent to about a fifth of that given by an equal weight of *N*-acetylglucosamine.

Concentration of p-*dimethylaminobenzaldehyde.* The influence of the concentration of *p*-dimethylaminobenzaldehyde in the DMAB reagent on the intensity of the colour formed was investigated by the standard procedure. The DMAB reagents used in the test series contained 1, 2 and 4% (w/v) *p*-dimethylaminobenzaldehyde. The results indicated that increasing concentrations of *p*-dimethylaminobenzaldehyde in the Ehrlich's reagent give rise to a small increase in colour for a constant amount of the *N*-acetylhexosamine. The advantage gained by the development of an increased intensity of colour when using a more concentrated solution of DMAB, however, is offset to some extent by a corresponding increase in the correction necessary to compensate for the enhanced yellowish green coloration given by the reagent's 'blank'.

Absorption spectrum of the N-*acetylhexosamines after alkali treatment and the addition of DMAB reagent*

Amounts of *N*-acetylglucosamine (20 μg.) and *N*-acetylchondrosamine (100 μg.), selected so that solutions of approximately the same colour intensity would finally result, were treated according to the standard procedure and, after standing for 1·5 hr. at 20°, examined in the spectrophotometer over the wavelength range 350–610 mμ. Control solutions were treated in the same manner except that the *N*-acetylhexosamine and alkali mixture was not heated and no colour developed. The results are given in Figs. 4 and 5 and show that the *N*-acetylhexosamines give rise to a coloured complex with *p*-dimethylaminobenzaldehyde which shows light absorption with maxima at 550 and 590 mμ. and a minimum at about 570 mμ. No absorption peaks were observed between 350 and 500 mμ. and this part of the curve is omitted in Figs. 4 and 5.

The rate of development and fading of the colour was also followed, and it was observed that the maximum intensity of

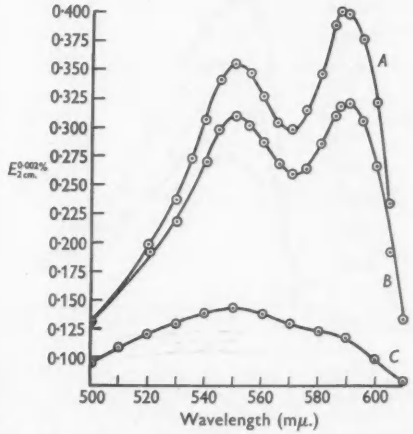

Fig. 4. Absorption spectrum of coloured complex derived from *N*-acetylglucosamine after treatment with Na_2CO_3 and DMAB reagent. Absorption after *A*, 1·5 hr., *B*, 4 hr. and *C*, 24 hr.

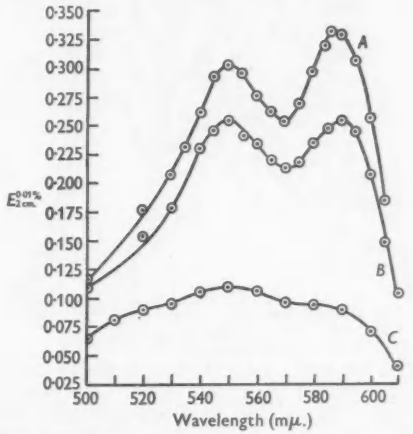

Fig. 5. Absorption spectrum of coloured complex derived from *N*-acetylchondrosamine after treatment with Na_2CO_3 and DMAB reagent. Absorption after *A*, 1·5 hr., *B*, 4 hr. and *C*, 24 hr.

Effect of concentration of NaCl *on the colour obtained.* To a series of tubes each containing *N*-acetylglucosamine (20 μg. in 0·1 ml.) and 0·2 ml. of 0·125 N-Na_2CO_3, was added (*a*) water, (*b*) 1% NaCl, (*c*) 3% NaCl, (*d*) 5% NaCl or (*e*) 10% NaCl to yield a total volume of 1·1 ml. The resulting solutions were then treated by the standard procedure and the intensity of the colours finally obtained were read on the photoelectric colorimeter 0·5, 1, 1·5, 2 and 24 hr. after the addition of the DMAB reagent. The concentrations of NaCl used in the experiments (*b*) and (*c*) gave rise to no appreciable change in the rate or amount of colour obtained as compared with that which arises using the 'standard' conditions. The colour development in the presence of the NaCl concentrations (*d*) and (*e*) was slower, and the maximum colour intensity obtained was less.

absorption of both peaks (550 and 590 mμ.) was reached at 20° in about 1·5 hr. after the addition of DMAB reagent. On standing the colour fades, but the rate of fall in the absorption intensity at 590 mμ. was faster than that at 550 mμ. The absorption intensity observed after the coloured solutions had stood for 4 hr. and 24 hr. for each *N*-acetylhexosamine is given in Figs. 4 and 5.

The two absorption peaks could be due to the interaction of one complex chromogenic structure or of two distinct chromogens with the *p*-dimethylaminobenzaldehyde. In order to determine if one of the absorption maxima arises from the presence of an intermediate product of the action of alkali on *N*-acetylglucosamine, specimens of this substance were heated at 100° for 1, 2, 4, 8 and 12 min. with 0·1 ml. of 0·5 N-Na_2CO_3, treated with glacial acetic acid and DMAB reagent in the usual manner and allowed to stand for 1·5 hr.

at 20°. The absorption curves obtained for a selected number of heating times, Fig. 6, demonstrate the similarity of the absorption after the different periods of heating, and show that the extent of heat treatment is reflected equally in the intensity of the absorption at 550 and at 590 mμ. Similar results were obtained with *N*-acetylchondrosamine.

The coloured products obtained by heating *N*-acetylglucosamine and *N*-acetylchondrosamine with different buffer systems at 100°, such as with potassium borate (pH 11) or glycine buffer (pH 10·9) in place of Na_2CO_3, and subsequent treatment with the DMAB reagent, have the same absorption characters. In order to obtain a maximum colour with these buffers, however, it was necessary to heat the borate and glycine buffer systems for 7 and 40 min., respectively, in place of the 4 min. heating required when Na_2CO_3 was employed (see Table 1).

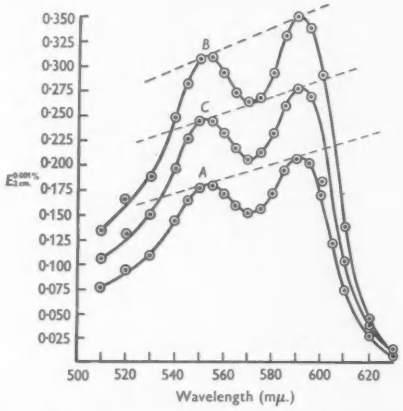

Fig. 6. Effect of varying the time of heating *N*-acetylglucosamine with Na_2CO_3 on the absorption spectrum of the coloured complex formed with DMAB. *A*, 2 min. heating; *B*, 4 min. heating; *C*, 8 min. heating.

The green filter used in the photoelectric colorimeter employed for the routine measurement of the intensity of the colour in the quantitative determination of the *N*-acetylhexosamines gives a maximum transmission of 42% at 550 mμ. whilst at 570 and 590 mμ. the transmissions are about 34 and 20%, respectively. Thus the filter measures predominantly the intensity of absorption at 550 mμ.

The exact amount of colour given by *N*-acetylchondrosamine in terms of *N*-acetylglucosamine was determined by measuring the colour developed from a series of standard amounts of *N*-acetylglucosamine (50, 60, 70 and 80 μg.) and from 300 μg. of *N*-acetylchondrosamine. Measurements were made on the Uvispek spectrophotometer at 550 and 590 mμ. using cells 2 cm. deep. The results showed that at 550 mμ. 71±2 μg. of *N*-acetylglucosamine gave a solution of the same colour intensity as 300 μg. of *N*-acetylchondrosamine. At 590 mμ. 68±2 μg. of *N*-acetylglucosamine were equivalent to 300 μg. of *N*-acetylchondrosamine.

Ultraviolet absorption of the N-acetylhexosamines after heating with alkali

N-Acetylglucosamine (300 μg. in 3 ml. water) was run into a 15 ml. volumetric flask, 1·5 ml. of 0·5N-Na_2CO_3 was added and the contents immediately made up to 15 ml. Half of the solution was heated for 4 min. at 100° and cooled; the remainder was unheated. The solutions were examined at once in the Uvispek spectrophotometer over the wavelengths 215–270 mμ., the unheated portion of the solution, which itself showed a strong absorption at 217 mμ., being used as the reference solution. The results are given in Fig. 7 and show that there is only a single absorption maximum and that this occurs at about 230 mμ. The intensity of absorption at this wavelength is greatest after 4 min. heating, that is, after the time of heating necessary to yield subsequently the maximum colour following the addition of the DMAB reagent. The intensity of the absorption at 230 mμ. is directly proportional to the amount of

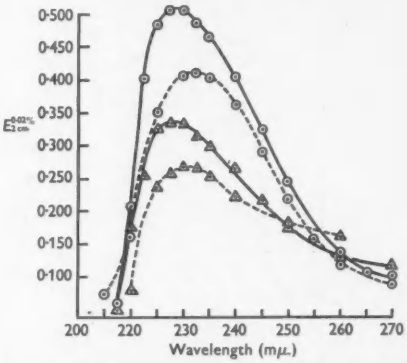

Fig. 7. Absorption in the ultraviolet region of *N*-acetylglucosamine and *N*-acetylchondrosamine after heating with Na_2CO_3. *N*-Acetylglucosamine, ⊙—⊙, after about 20 min.; ⊙ - - - - ⊙, after 4 hr. *N*-Acetylchondrosamine, △—△, after about 20 min.; △- - - -△, after 4 hr.

N-acetylglucosamine heated with alkali and decreases on standing, with a slight shift in the position of the absorption maximum toward a longer wavelength. The absorption recorded after the alkali-treated *N*-acetylhexosamine solution had stood for 4 hr. at room temperature is also shown in Fig. 7. It is evident that the chromogenic structure is alkali-labile. The immediate addition of the DMAB reagent after the optimal time of heating with alkali is essential if maximum colour production is to be obtained subsequently in the colorimetric determination.

N-Acetylchondrosamine treated with dilute Na_2CO_3 under conditions identical with those described gives a similar absorption curve (Fig. 7) which likewise shows a maximum absorption at about 230 mμ. It is to be noted, however, that the maximum intensity of the absorption at 230 mμ. for *N*-acetylchondrosamine was considerably less than that given by an equal quantity of *N*-acetylglucosamine.

Solutions (20 μg./ml.) of methyl-L-fucopyranoside, *N*-methyl-L-glucosamine hydrochloride, *p*-tolyl-D-*iso*glucosamine, *N*-acetylglucosaminol, methyl-*N*-acetylglucosaminide, L-fucose, D-glucose, D-galactose, L-rhamnose, digitoxose, L-fucodesose, lactose, *N*-acetyl-1-aminoglucose and D-ribose were treated with alkali under the same conditions, but none of these substances gave an absorption curve similar to that obtained with the *N*-acetylhexosamines with a single maximum absorption at 230 mμ.

The absorption spectrum of glucose-lysine mixtures after alkali treatment and after alkali treatment and the addition of DMAB reagent

The finding by Vasseur & Immers (1949) that mixtures of certain amino-acids, especially lysine, and simple sugars develop, after treatment with alkali and DMAB reagent, a colour similar to that given by the *N*-acetylhexosamines, suggested that the visible and ultraviolet absorption characteristics of lysine-glucose mixtures should be examined.

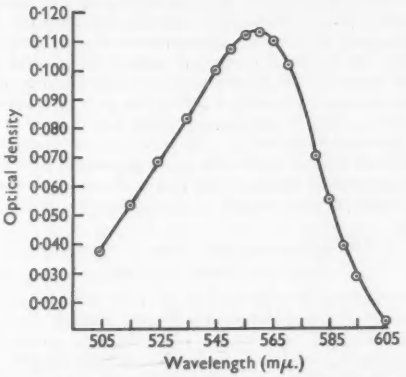

Fig. 8. The absorption spectrum of a mixture of glucose and lysine after treatment with Na_2CO_3 and DMAB.

Glucose (30 mg.) and lysine (15 mg.) were added separately and together to 1·5 ml. quantities of 0·5 N-Na_2CO_3 and the total volume in each instance was immediately made up to 15 ml. with water. The three solutions were divided into equal parts and one half of each was heated for 4 min. at 100° and then cooled. The solutions of glucose and of glucose + lysine developed a yellow colour, the solution of lysine remained colourless. The ultraviolet absorption of the solutions was then determined using the unheated portions as reference solutions. The results indicated that only the heated glucose and the heated glucose-lysine mixture showed any appreciable general absorption and in both instances the characteristic absorption maximum at 230 mμ. given by the *N*-acetylhexosamines was absent.

The heated and unheated test solutions (1 ml. of each) were then treated with glacial acetic acid and DMAB reagent (1 ml.) according to the usual procedure. The yellowish brown coloration of the reaction mixtures which contained heated glucose and glucose-lysine mixture disappeared on the addition of glacial acetic acid. The heated and unheated glucose and lysine solutions and the unheated glucose-lysine mixture developed a pale-yellow colour only, whereas the heated glucose-lysine mixture (3 mg.) slowly developed a pink coloration. The absorption curve of the coloured solution is given in Fig. 8 and shows only a single absorption maximum at about 560 mμ.

Absorption spectra of the blood-group substances after treatment with (a) alkali and (b) alkali and DMAB reagent

The human blood-group mucoids after treatment with alkali and DMAB reagent give rise to a colour which appears to be identical with that formed by *N*-acetylglucosamine after similar treatment. It is possible, however, in view of

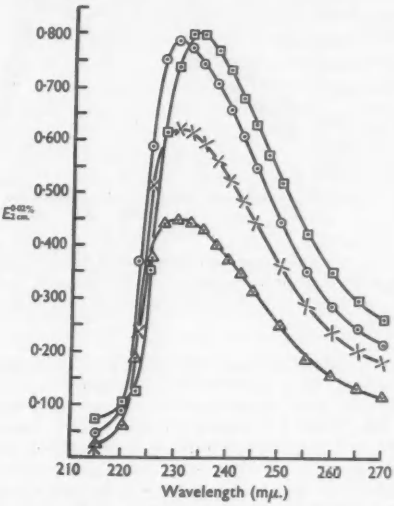

Fig. 9. Absorption in the ultraviolet of blood-group mucoids after treatment with dilute alkali at 100°, ⊙—⊙, A substance; ⊡—⊡, B substance; ×—×, H substance; △—△, Le^a substance.

the observations of Vasseur & Immers (1949) that several component molecules of the group mucoids other than the *N*-acetylhexosamines, such as galactose and fucose and the individual amino-acids, especially if free, will combine in alkaline solution to form a substance which will give a coloured complex with DMAB. It became of interest, therefore, to examine not only the amount of colour given, but also the absorption characteristics of the alkali-treated blood-group substances and coloured products obtained from them on the addition of the DMAB reagent.

Alkali alone. The group substances A, B, H and Le^a (3 mg. in 1·5 ml. 0·5 N-Na_2CO_3) were each made up to 15 ml. with water and divided into two approximately equal parts. One part was heated at 100° for 12 or 15 min. (see below), whilst the remainder of the solution was kept as an unheated control. The heated solution was cooled and examined immediately in the spectrophotometer, and its absorption with reference to the unheated material was measured over the wavelength range 215–270 mμ. The results obtained are given in Fig. 9 and show that in each

instance a structure is formed which gives a single absorption maximum at about 230 mμ. Solutions of equal concentration (200 μg./ml.) of dextran, glycogen, gum arabic, mannocarolose, galactocarolose, and the specific polysaccharides of *Shigella shigae* and pneumococcus Type II gave no comparable amount of absorption over the same wavelength range and showed no characteristic absorption maximum at 230 mμ. after treatment with alkali under the same conditions as were used for the group mucoids.

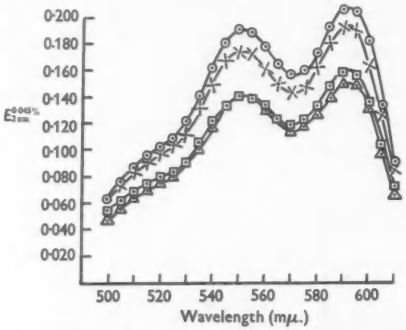

Fig. 10. Absorption spectrum of coloured complex derived from blood-group mucoids. ⊙—⊙, A substance; ⊡—⊡, B substance; ×—×, H substance; △—△, Le^a substance.

Alkali and DMAB reagent. The group substances (500 μg.) contained in 1 ml. water were heated with 0·1 ml. of 0·5 N-Na_2CO_3 for 12 or 15 min. (see below), cooled and treated according to the standard procedure. The absorption spectrum of each group substance was measured over the range 500–610 mμ. using the unheated group substance, which in no instance gave a purple colour under the test conditions, as the reference solution. Group mucoids so treated sometimes develop a slight opalescence which should be removed by the addition of 1 ml. water to all solutions just before the absorption intensities are measured. The results are given in Fig. 10 and indicate that absorption curves similar to those given by the *N*-acetylhexosamines were obtained. The points of maximum absorption intensity were again at about 550 and 590 mμ. with a minimum at 570 mμ.

The influence of the time of heating of the group mucoids with alkali on the intensity of the colour subsequently obtained on the addition of the DMAB reagent was investigated using the 'standard' procedure, and the results obtained (Table 2) in one instance (A substance) showed that the optimal period of heating is 12 min. Somewhat longer times, about 15 min., were required by B and H substances. It seems that a minimum time of 12 min. heating should be employed when measuring the '*N*-acetylhexosamine' colour given by the group mucoids. The polysaccharides used as control materials and mentioned above give no colour after heating with alkali for 15 min. and the addition of the DMAB reagent.

The amount of colour obtained with A substance, after treatment with dilute Na_2CO_3 or one of the other buffers, is recorded in Table 2 where the results are expressed in terms of the absorption given by an equal weight of *N*-acetylglucosamine. The values obtained may be compared with those given for *N*-acetylglucosamine or *N*-acetylchondrosamine (Table 1) and show that heating the A substance with borate buffer gives rise to a material which shows about the same intensity of absorption as is obtained with glycine, or Na_2CO_3, in contrast to the behaviour of the *N*-acetylhexosamines which give an enhanced colour when heated in borate buffer. The group substances give maximum colour intensities equivalent to the absorption given by 7–10% of their weight of *N*-acetylglucosamine.

The influence of oxidation of A substance on the absorption spectrum

Oxidation with sodium periodate. A maximum intensity of absorption was observed at 230 mμ. with A substance oxidized with periodate at pH 5 for 7 hr. (Aminoff & Morgan, 1951) and treated with alkali alone. The addition of the DMAB reagent gave rise to the production of a purple colour which showed two absorption maxima, at 550 and 590 mμ. A quantitative determination of the amount of '*N*-acetylglucosamine' colour given by the oxidized A substance showed that there was probably no significant

Table 2. *Amount of colour given by blood-group A substance after treatment at* 100° *with different buffer systems and the addition of DMAB reagent*

Alkaline system	pH	Period of heating for maximum colour production (min.)	Amount of colour expressed as % of amount given by *N*-acetylglucosamine* (Observed)	(Extrapolated value)
0·1 ml. 0·125 N-Na_2CO_3	10·8	35	7·8	9·2
0·4 ml. 0·125 N-Na_2CO_3	10·8	12	6·8	9·1
0·8 ml. 0·125 N-Na_2CO_3	10·8	10	8·4	10·7
Potassium borate + KOH	9·0	†	—	—
	10·0	80	6·2	7·1
	11·0	45	6·2	7·9
Glycine buffer	10·0	†	—	—
	10·9	150	7·1	8·5
	12·2	5	6·2	7·9

* Under 'standard' conditions. † Maximum colour not reached after 4 hr. heating.

change in this property as a result of oxidation with periodate. In these respects, therefore, the oxidized A substance behaves as does the untreated blood-group substance.

Oxidation with hypoiodite. The procedure described by MacLeod & Robison (1929) was used. A substance (5 ml. of 0·5%) was added to 3·0 ml. of 0·1 N-I_2 and 2·0 ml. of 0·5 N-Na_2CO_3 contained in a 50 ml. glass-stoppered flask. The solution was kept at 22° in the dark for 7 hr., after which time oxidation was essentially complete and the serological activity of the A substance was largely destroyed. The oxidized mucoid was dialysed against distilled water at 0°, made up to a known volume and 500 μg. were treated with alkali according to the conditions described in the standard procedure for the determination of *N*-acetylglucosamine. The oxidized and alkali-treated material showed a maximum absorption at 230 mμ., but the intensity of absorption was less than that given by an equal quantity of unoxidized material. The addition of the DMAB reagent gave rise to a purple-coloured solution which showed two absorption maxima, at 550 and at 590 mμ. A quantitative measurement of the '*N*-acetylglucosamine' colour revealed that the A substance after oxidation with hypoiodite gave rise to about 80% of the colour intensity reached by the original A substance. A detailed account of the oxidation of the blood-group mucoids with hypoiodite will be given elsewhere.

DISCUSSION

The conditions usually employed for the colorimetric determination of the *N*-acetylhexosamines have been re-examined in some detail, and it is evident from the results obtained that these substances undergo rapid structural change on treatment for a short time with hot dilute alkali or alkaline buffers, and that in each instance a substance is formed which shows strong light absorption between 220 and 250 mμ. with a maximum absorption at about 230 mμ. The gradual disappearance of the absorption maximum observed, however, indicates that the substance formed by the action of alkali is itself unstable in alkaline solution.

The substances formed from the *N*-acetylhexosamines by the action of dilute alkali condense readily with *p*-dimethylaminobenzaldehyde in acetic acid to yield a solution of intense purple colour which shows characteristic absorption maxima at 550 and 590 mμ. with a minimum at about 570 mμ. (cf. Osaki & Turumi, 1947; Gottschalk & Lind, 1949). In view of the unstable nature of the chromogenic material, it is essential for the quantitative determination of *N*-acetylhexosamine that the *p*-dimethylaminobenzaldehyde reagent should be added and the test completed immediately the solutions under examination have been heated with alkali.

Treatment of the *N*-acetylhexosamines with alkali under different conditions has revealed that increasing the concentration of Na_2CO_3 results not only in a more rapid formation of the chromogenic structure, but also brings about its more rapid destruction and, although the period of heating necessary to yield the maximum colour after the addition of the *p*-dimethylaminobenzaldehyde reagent becomes shorter with increasing alkali concentration, the actual amount of colour obtained decreases with higher concentration of alkali. Extrapolation of that part of the curve which measures the rate of destruction of the chromogenic material gives a corrected value for the maximum colour intensity found. The corrected value obtained, however, is of no great accuracy. It will be noticed that the alkali concentration finally recommended as suitable for the quantitative determination of the *N*-acetylhexosamines is not that which gives rise to the minimum destruction of the chromogenic substances and consequently to greatest colour intensity per unit weight of the *N*-acetylhexosamines. The alkali concentration finally chosen was selected so that any slight buffer action of the solution under test would not alter significantly the pH of the solution during treatment with alkali which should be close to pH 10·8. Treatment of the *N*-acetylhexosamines with borate buffer at pH 10·8 gives rise to an enhanced colour production as compared with that obtained using sodium carbonate at the same pH.

The interaction in an alkaline medium of simple hexoses and certain amino-acids with the formation of a substance that gives with *p*-dimethylaminobenzaldehyde reagent a colour which closely resembles that given by *N*-acetylglucosamine after similar treatment was first reported by Vasseur & Immers (1949) and subsequently confirmed by Gottschalk & Partridge (1950). The light absorption given by a mixture of lysine and glucose after treatment with dilute alkali has now been determined, and it has been found that within the range 210–300 mμ. there is no characteristic absorption maximum similar to that given by the *N*-acetylhexosamines. The purple-coloured solution which is formed on the addition of the *p*-dimethylaminobenzaldehyde reagent to the alkali-treated glucose-lysine mixture shows a single absorption maximum at 560 mμ. and not two maxima at 550 and 590 mμ. as is shown by the *N*-acetylhexosamines after identical treatment. The product of the interaction of glucose and lysine, therefore, although giving a reddish purple colour with *p*-dimethylaminobenzaldehyde is nevertheless readily distinguished from that which arises from the *N*-acetylhexosamines. The colour given in the 'Direct Test' of Osaki & Turumi (1947) appears to be due to an amino-acid-sugar complex and not, as they suggest, to a different ring structure associated with *N*-acetylhexosamine.

Morgan & King (1943) reported that a purified mucoid which was obtained from hog gastric mucin and which possessed intense human blood-group A activity, gave a colour reaction after treatment with

dilute alkali and *p*-dimethylaminobenzaldehyde similar to that given by *N*-acetylglucosamine. Subsequently it was suggested (Morgan & Waddell, 1945; Morgan, 1947) that this colour reaction was a characteristic property of the blood-group mucoids. It is now known that solutions of essentially homogeneous preparations of the blood-group mucoids which are responsible for the group characters A, B, the recently discovered 'Lewis' Le^a property and the so-called O (H) character of the tissue fluids and secretions, all develop a purple colour after treatment with dilute alkali and the addition of the *p*-dimethylaminobenzaldehyde-hydrochloric acid reagent. In each instance the maximum light absorption of the coloured solution is found to be at about 550 and 590 mμ., whereas the solutions of the material after alkali treatment alone show a single absorption maximum at 230 mμ. In both these respects, therefore, the group substances behave as do the *N*-acetylhexosamines.

The blood-group substances require a minimum of about 12 min. heating with alkali to yield the maximum amount of colour with the *p*-dimethylaminobenzaldehyde reagent in place of the 4 min. heating necessary for the *N*-acetylhexosamines. The intensity of the colour given by the group substances under these conditions varies somewhat for each group substance investigated, but is found to be equivalent to between 7 and 10% of the colour intensity given by an equal weight of *N*-acetylglucosamine. It is to be noted that there is no enhancement of colour production when a borate buffer of the same pH value is employed.

Group A substance rendered serologically inactive by oxidation with sodium periodate at pH 5 continues to yield a strong purple colour after treatment with alkali and the addition of DMAB reagent (Aminoff & Morgan, 1951). It is now shown that A substance after oxidation with periodate and treatment with alkali shows a single absorption maximum at 230 mμ. and that the purple colour which subsequently arises after the addition of the DMAB reagent also shows absorption maxima of undiminished intensity at 550 and 590 mμ. The A substance after oxidation with periodate therefore behaves as does the unoxidized material. Aminoff & Morgan (1951) considered that oxidation of the A substance with periodate left unchanged the reducing group and the *N*-acetyl group of the *N*-acetylchondrosamine end unit, to allow for the formation of the chromogenic structure on treatment with alkali, and the results of the absorption studies now reported with A substance oxidized with periodate appear to support this suggestion.

Oxidation of the A substance with sodium hypoiodite, on the other hand, forms a substance which gives a reduced '*N*-acetylglucosamine' colour value after alkali treatment and the addition of *p*-dimethylaminobenzaldehyde. It seems probable that oxidation of the A substance with hypoiodite converts the aldehyde group of the *N*-acetylchondrosamine residue present as a reducing end group into a carboxyl group. Such a change would result in the end residue being unable to form a chromogenic structure on treatment with alkali and in consequence the oxidized A substance would yield with *p*-dimethylaminobenzaldehyde a solution of diminished colour intensity. As *N*-acetylchondrosamine gives about one-fifth of the amount of colour given by *N*-acetylglucosamine it must be concluded that *N*-acetylglucosamine contributes to the total colour given by the A substance. Aminoff & Morgan (1949, 1951) suggested that the terminal *N*-acetylchondrosamine residue is linked to C atom 1 of its neighbouring *N*-acetylglucosamine residue and that treatment of the A substance with alkali under the conditions used for the determination of *N*-acetylhexosamine hydrolyses this glycoside linkage and thus transforms the penultimate *N*-acetylglucosamine into a reducing end group and renders it susceptible to the action of alkali with the consequent formation of a chromogenic structure which gives a coloured complex with *p*-dimethylaminobenzaldehyde (cf. Morgan, 1946). If the total colour given by the A substance after treatment with alkali and *p*-dimethylaminobenzaldehyde arises in this way from *N*-acetylchondrosamine and *N*-acetylglucosamine units then the decrease in the colour intensity to be expected from A substance after treatment with hypoiodite will be about 18% of the original value expressed in terms of *N*-acetylglucosamine. A decrease of this order has been found. The action of alkaline hypoiodite and of dilute alkali on such complex materials as the blood-group mucoids is, however, far from straightforward, and the results of further work must be awaited before the changes brought about can be fully understood or the results obtained used to establish the presence of definite structures within the blood-group mucoids.

SUMMARY

1. The conditions for the colorimetric determination of the *N*-acetylhexosamines have been reinvestigated.

2. The period of heating necessary, and the maximum amount of colour obtained, when *N*-acetylglucosamine is treated with alkali and the *p*-dimethylaminobenzaldehyde reagent, depends not only on the pH of the system but also on the nature of the buffer employed. Borate buffer gives an enhanced colour production.

3. *N*-Acetylchondrosamine behaves similarly, but the intensity of the colour given is about 23%

of that obtained with an equal weight of *N*-acetylglucosamine.

4. The *N*-acetylhexosamines after treatment with alkali show a single maximum absorption at 230 mμ. and after the addition of the *p*-dimethylaminobenzaldehyde reagent the purple colour which develops shows two absorption maxima, at 550 and at 590 mμ.

5. A mixture of glucose and lysine heated with sodium carbonate shows no characteristic absorption maximum at 230 mμ. The purple colour obtained on the subsequent addition of *p*-dimethylaminobenzaldehyde reagent shows a single absorption maximum at about 560 mμ.

6. The human blood-group mucoids yield between 7 and 10 % of the colour intensity given by an equal weight of *N*-acetylglucosamine, and there is no enhanced colour production when a borate buffer is employed. The absorption characteristics of the chromogenic substances which arise from the group substance after heating with alkali, and of the coloured product obtained on the addition of the *p*-dimethylaminobenzaldehyde reagent support the belief that the colour given by the group substances arises from reducing *N*-acetylchondrosamine end groups and closely linked *N*-acetylglucosamine residues, which are readily liberated by the action of alkali.

7. The A substance, after oxidation with hypoiodite, gives rise to a coloured complex on treatment with alkali and *p*-dimethylaminobenzaldehyde which shows a reduced absorption intensity compared with that given by the unoxidized material. A substance oxidized with periodate and treated under similar conditions shows no decrease in absorption. The significance of these findings is discussed.

The investigation was supported by personal grants made to two of the authors (W.M.W. and D.A.) by the Medical Research Council and the Governing Body of the Lister Institute respectively. The authors are indebted to the University of London Research Fund for a grant to purchase the Uvispek spectrophotometer used. We wish to acknowledge our indebtedness to Miss V. Hayes and Miss J. Pugh for valuable technical assistance.

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A Study of the Breakdown of Ribonucleic Acid in Tobacco-leaf Extracts

By G. PARKER*
Rothamsted Experimental Station, Harpenden, Herts

(*Received* 18 *October* 1951)

There is an extensive literature on the preparation of ribonucleic acids and their degradation products from animal tissues and micro-organisms. By contrast, apart from the isolation of certain plant virus nucleoproteins, little work has been undertaken until recently, on the preparation of ribonucleic acids from plant material.

* Present address: The Distillers Co. Ltd. Speke, Liverpool 19.

Foreman (1938) suggested the presence of nucleoprotein in leaves of perennial rye grass, to explain the fact that cytolysed leaves yielded only 30 % of their phosphorus on extraction with boiling water, whereas leaves previously dried at 85° yielded all their phosphorus by cold water extraction. The occurrence in, and isolation of, ribonucleic acid from leaves of barley, rye and spinach has been reported by von Euler & Hahn (1947, 1948). These

workers used an alkaline extraction method similar to that employed by Schmidt & Thannhauser (1945) for animal tissues. Takasugi (1943, 1944) used 10% sodium chloride solution to extract the nucleic acids from barley roots. Ogur & Rosen (1950) used perchloric acid for the separate extraction of ribo- and deoxyribo-nucleic acids from corn root tips.

Pirie (1950) has described the isolation of a ribonucleoprotein from the sap of the leaves of healthy tobacco plants. As prepared, this material, whose concentration in sap varies with the age of the leaf, is associated with enzymes (ribonuclease and phosphatase) capable of bringing about its degradation. Pirie was able to obtain the nucleic acid from fresh preparations of this nucleoprotein by denaturation of the protein with trichloroacetic acid (TCA), but he could only isolate degradation products of the acid after such precipitation from sap which had not been ultracentrifuged. It appeared of interest to examine these degradation products more closely, and the work reported in the present paper deals with the preparations of various phosphorus-containing fractions obtained from sap by chemical and enzymic degradation of the nucleic acid moiety of the trichloroacetic acid sap precipitate which contains this nucleoprotein.

MATERIALS AND GENERAL PROCEDURES

Preparation of sap-trichloroacetic acid precipitate and of chloroplast-trichloroacetic acid precipitate

Leaves, between 10 and 20 cm. long, of tobacco (*Nicotiana tabacum* var. White Burley) from plants grown in a heated glasshouse were used for most of the work. Single experiments confirming the results obtained with tobacco have also been carried out with the leaves of turnip, spinach, French bean, comfrey, snowberry and grass, and oat and barley seedlings. Unless stated otherwise, results quoted in the text refer to tobacco. The leaves were taken at all periods of the year; different batches consequently probably differed in age, but no attempt has been made to analyse the results for possible seasonal variations in composition.

The midribs were removed from the leaves and the laminae passed through a domestic mincer and squeezed on madapollam into a receiver cooled in ice. The residue was re-minced and squeezed again. Cell debris, chloroplasts and starch granules were removed by centrifuging for 15 min. in a chilled angle centrifuge at 8000 rev./min. (6400*g*). The supernatant fluid, which is subsequently referred to as sap, was stored at 4° and used within 30 min. of preparation. Unless otherwise stated, it was treated with 50% (w/v) trichloroacetic acid (TCA) to a final concentration of 5%. The precipitate was centrifuged down at once and washed several times, first with 2% (w/v) TCA and then with water, the volume of wash liquor in each case being about half that of the sap, until the washings contained only 1–2 μg. P/ml. The solid residue is subsequently termed sap-TCA precipitate.

The chloroplasts, separating from crude sap as a sticky layer overlying the starch, were roughly separated from the latter by scraping with a spatula. They were washed twice with distilled water and were then dispersed to give a suspension which was used immediately after preparation. It was precipitated with TCA and washed in the same way as the sap, and is referred to subsequently as chloroplast-TCA precipitate.

Analytical methods

Phosphorus. This was determined by a modification of the method of Kuttner & Lichtenstein (1932). Inorganic phosphate values were obtained on samples which had not been incinerated. P retained by samples containing Ba was estimated by incineration with H_2SO_4 until the sample dissolved completely: after dilution, the precipitate was centrifuged down, and the inorganic phosphate content of the supernatant liquid measured.

Ribose. Total ribose estimations were made by the orcinol method of Pirie (1936) using ribose standards. The colour developed was measured in a photoelectric absorptiometer (the Evans Electroselenium Portable model was used) using Ilford filter 622.

Values for purine-bound ribose were obtained by the Markham & Smith (1949) modification of Militzer's (1946) orcinol method. The colour developed was read in the absorptiometer against that due to ribose standards, using Ilford filter 205.

Purines. These were determined in N-H_2SO_4 hydrolysates by the following methods.

Adenine. This was estimated colorimetrically by Woodhouse's (1950) method, modified by the use of twice the concentration of ammonium sulphamate recorded by Woodhouse. This avoided difficulties, otherwise encountered, through incomplete decomposition of excess nitrite, which led to production of cloudy solutions.

Guanine. The method of Williams (1950) was employed. Standard guanine solutions were subjected to precipitation along with the samples, and the colour developed with the phenol reagent was read, using Ilford filter 608.

Total pyrimidines. These were obtained in the free form by the method of Daly, Allfrey & Mirsky (1950), and were then determined by the colorimetric procedure of Soodak, Pircio & Cerecedo (1949) using standard uracil solutions and Ilford filter 608.

Phosphatase activity. This was determined using, as substrate, solutions of sodium β-glycerophosphate and metaphosphate, and yeast adenylic acid. The reaction mixture comprised substrate solution at pH 6, 0·02 M-sodium citrate buffer, pH 6, enzyme solution or suspension, and water to a total volume of 5 ml., and contained 100 μg. P/ml. It was incubated at 37°. Samples were withdrawn at intervals and pipetted into an equal volume of 10% (w/v) TCA: after centrifuging at 1500 rev./min. for 10 min., the supernatant fluid was poured off and analysed for inorganic phosphate. Control reaction mixtures, from which the enzyme preparations and the substrate were individually omitted, were set up initially. They were found to give such low blank values as to be unnecessary, and were omitted in the later experiments.

Ribonuclease activity. This was measured by determining the amount of P becoming soluble in the uranyl nitrate and TCA reagent of McFadyen (1934) after action of the enzyme preparation on yeast ribonucleic acid. The reaction mixture comprised a solution of the acid brought to pH 6, purified by

the method of Vischer & Chargaff (1948*b*), 0·02 M-sodium citrate buffer, pH 6, enzyme solution or suspension, and water to a total vol. of 5 ml., and contained 100 μg. P/ml. It was incubated at 37°. Samples were removed at intervals into 1 ml. of the uranyl reagent, chilled at 0° for 15 min. and then centrifuged at 1500 rev./min. for 10 min.; the supernatant fluid was poured off and an appropriate volume taken for total P analysis. As in the case of the phosphatase determinations, control reaction mixtures omitting enzyme or substrate were found to be unnecessary, and were only set up in a few preliminary experiments.

The optimum pH for nuclease activity was obtained in a similar reaction mixture, using the Michaelis (1931) veronal-acetate buffer (1·0 ml. of the diluted stock solution in 5 ml. total reaction mixture) of appropriate pH. The pH of the reaction mixture was determined at the beginning and the end of the experiment, but showed no signs of drift during the reaction at any pH employed.

Paper chromatography. Purines and pyrimidine nucleotides were separated on Whatman no. 1 or no. 11 paper using the *tert.*-butanol-HCl solvent system of Smith & Markham (1950). They were located on the paper by the mercury reagents of Vischer & Chargaff (1948*a*) and by viewing in suitably filtered ultraviolet light (Holiday & Johnson, 1949).

Ultraviolet absorption spectra. These were obtained with a Hilger absorption spectrograph.

pH *measurements.* These were made with a glass electrode.

RESULTS

Fractionation of sap-TCA precipitate: preparation and properties of mononucleotide solutions

Sap-TCA precipitate, prepared as described, was mixed with water to a thin paste, cooled to 0° and brought to pH 9 with 0·5 N-NaOH. Partial solution occurred. After standing overnight at 4°, the mixture was centrifuged at 8000 rev./min. (6400*g*) and the supernatant decanted. The residue was re-extracted with NaOH, pH 9, at 4° for several hours, again centrifuged down and then washed with a little water. The combined dark brown extracts, the total volume of which was approximately one-third that of the original sap, contained much of the protein originally present in the sap-TCA precipitate. This was thrown out by progressive acidification to a pH of approximately 6, 5 and 4. At each pH the precipitate was centrifuged down and washed with a little water, the washings being added to the main supernatant before the pH was readjusted. The almost colourless pH 4 supernatant contained no nucleic acid as it gave no precipitate on acidifying to pH 1 or less. It was treated with saturated $Ba(OH)_2$ solution to pH 9 and stood overnight at 4°. Any precipitate separating was centrifuged out, and the nucleotides were precipitated from the supernatant with 3 vol. of 95% ethanol at 4°. The barium salts were dissolved in the minimum volume of water and decomposed with 0·5 N-H_2SO_4 to pH 3. Precipitated $BaSO_4$ was centrifuged down and washed with a little water, and the combined supernatants were treated with a little $Ba(OH)_2$ solution and quantitatively freed from Ba^{++} ions with 5% (w/v) Na_2SO_4 before finally centrifuging clear. The aqueous ethanolic supernatant remaining after separation of the barium nucleotides usually yielded a second crop of barium salts when treated with more $Ba(OH)_2$ solution and stored at 4° for 24 hr.: these were decomposed with H_2SO_4 in the same way.

In Table 1 is given the distribution of P between the residue insoluble at pH 9, the nucleotide preparations and the protein precipitates in the above procedure. These three fractions comprise the bulk of the P originally present in the sap-TCA precipitate. The remaining 10% was mostly found

Table 1. *Distribution of phosphorus in some fractions of sap*

Fraction	P, as % of total of: Sap	Sap-TCA precipitate	pH 9 extracts
pH 9-insoluble residues	1–3	5–15	—
Mononucleotide preparations	7–10	40–55	50–65
Protein precipitates	3–8	20–35	20–40

associated with the $BaSO_4$ precipitates obtained by decomposition of the barium nucleotides. Small amounts were also found in the precipitates sometimes obtained with Ba^{++} at pH 9 before addition of ethanol, and in the aqueous ethanolic supernatants remaining after precipitation of the 'second crop' nucleotides. In this way it is possible to account for a total of over 95% of the P of the sap-TCA precipitate.

In a single experiment, several of the aqueous ethanolic solutions remaining after precipitation of two crops of nucleotides were pooled and evaporated to one-tenth volume in vacuum, but attempted precipitation of further nucleotides by $Ba(OH)_2$ and ethanol yielded no barium-soluble, ethanol-insoluble material.

The figures in Table 1 vary somewhat with each batch of sap. This is not surprising. Variations are to be expected from causes such as differences in age of leaves taken at various times of the year, the use of different samples of soil in growing the plants, and unavoidable variations in the experimental technique with different lots of leaves. The values given are the extreme ranges obtained with several different batches of sap.

No precipitate was produced in the preparations by solutions of uranyl nitrate in TCA at a pH of less than 1, but addition of alkali caused mononucleotides to be thrown down, maximum visible precipitation occurring in the range pH 1·5–2 (cf. Zittle, 1946). Treatment with picric acid had no effect, but Buell's (1943) aluminium picrate reagent gave immediate yellow precipitates containing the adenylic acid and most of the guanylic acid of the preparations. Pyrimidine nucleotides are not precipitated by this reagent but their occurrence was indicated by the presence of a substantial proportion (50–60%) of the initial P in the filtrates. Confirmation was obtained in later experiments, when paper chromatography of preparations after 60 min. hydrolysis in N-HCl showed the presence of the four components, adenine, guanine, cytidylic and uridylic acids, typical of ribonucleic acids.

Other experiments of the type described, all carried out on 'first crop' nucleotide preparations, indicated that these contained more pyrimidine than purine bases. This was supported by analysis of the preparations for purine-bound ribose, which indicated that this accounted for some 40% of the total carbohydrate. Confirmation was obtained by analyses of the preparations for purine and pyrimidine content. Typical results are shown in Table 2, which also

Table 2. *Purine and pyrimidine composition of two nucleotide preparations*

(In exp. 1, 1 l. sap contained 191 mg. P. It gave a sap-TCA precipitate containing 27·3 mg. P, and yielded 15·3 mg. P in nucleotide preparations. In exp. 2, the sap had 179 mg. P/l. and gave a sap-TCA precipitate containing 45·3 mg. P and yielded 17·7 mg. P in nucleotide preparations. 'First crop' indicates nucleotides obtained from the initial baryta-soluble, ethanol-insoluble precipitates; 'second crop' preparations resulted from addition of more $Ba(OH)_2$ to the supernatants from the 'first crop' precipitates. The composition of the nucleotide preparations is given as molecules base/100 atoms P.)

Exp.	Fraction	Adenine	Guanine	Total pyrimidines	P
		(molecules/100 atoms total P)			(atoms)
1	First crop	2·2	32·8	58·9	93·7
	Second crop	0·6	4·6	1·2	6·3
	Total	2·8	37·4	60·1	100
2	First crop	1·5	26·7	65·0	90·4
	Second crop	3·1	5·1	1·6	9·6
	Total	4·6	31·8	66·6	100

Table 3. *Effect of removal of lipids upon yields and composition of nucleotide preparations*

(Two equal portions of sap, from the same batch of leaves, were precipitated with TCA. The sap-TCA precipitates were fractionated for nucleotides as described in the text, one being treated with lipid solvents before fractionation. 'First' and 'second' crops have the meanings designated in Table 2. The composition of the preparations is given as molecules base/100 atoms P. Results of experiments on two separate lots of leaves are presented: (1) the sap (1 l., 231 mg. P) gave 45·5 mg. P in the sap-TCA precipitate. If lipids were not removed 21·2 mg. P were recovered in nucleotide preparations: if lipids were removed before fractionation, only 14·7 mg. P were so recovered; (2) the sap (1 l., 183 mg. P) gave 37·8 mg. P in the sap-TCA precipitate. This yielded 16·5 mg. P and 13·6 mg. P respectively as nucleotides following fractionation in presence and in absence of lipids.)

		Adenine		Guanine		Total pyrimidines		Phosphorus	
	Fraction	Sap 1	Sap 2	Sap 1	Sap 2	Sap 1	Sap 2	Sap 1	Sap 2
		(molecules/100 atoms total P)						(atoms)	
Lipids present	First crop	2·2	2·3	29·9	19·4	57·1	63·8	90·1	85·2
	Second crop	0·6	0·6	4·0	6·7	3·5	5·8	9·9	14·8
	Total	2·8	2·9	33·9	26·1	60·6	69·6	100	100
Lipids absent	First crop	0·4	0·2	32·5	30·4	42·7	52·8	78·2	86·9
	Second crop	3·9	1·7	10·5	6·4	7·0	4·9	21·8	13·1
	Total	4·3	1·9	43·0	36·8	49·7	57·7	100	100

shows that in the 'second crop' preparations this order is reversed and purines predominate. However, the total amount of pyrimidines in the 'first' and 'second' crops from any one batch of sap is always considerably greater than the total amount of purines.

Effect of ageing sap-TCA precipitate on phosphorus distribution

A small number of experiments was carried out on sap-TCA precipitates which had been left in contact with their TCA supernatants at room temperature for some hours before collection. The results were rather variable. However, the sap-TCA precipitate collected immediately after preparation usually contained somewhat more P than did the corresponding precipitate, from an equal volume of sap, allowed to age for some hours before it was collected. The difference increased the longer the precipitate was left to age. Aged precipitates yielded less P in the pH 9 extracts than did the fresh precipitates, but these extracts yielded protein precipitates which retained more P than did fresh sap-TCA precipitate extracts. Invariably, ageing of a sap-TCA precipitate led to reduction in the amount of P that was isolated in the nucleotide preparations.

Effect of removal of lipids from the sap-TCA precipitate upon yield and composition of the nucleotide preparations

Two equal volumes of a sap were precipitated with TCA and the precipitates washed in the usual manner. One precipitate was subjected to alkaline fractionation immediately. The other was extracted once with 95% ethanol and then with ethanol-ether (7:3, v/v) until the extracts were colourless: the residue was washed with water and then fractionated for nucleotides. Results of typical experiments are given in Table 3. This shows that removal of lipids from the sap-TCA precipitate lessened the amount of P which could be isolated as nucleotides. This was reflected in the total amount of pyrimidines present in the nucleotide preparations. The purine contents were affected to a very much smaller extent and the change may not be significant as it is difficult to ensure absolutely identical conditions of fractionation for each half of the batch of sap. The amount of P separating with the protein precipitates during fractionation was correspondingly greater in these cases than in the parallel experiments on precipitates from which the lipids were not removed. Removal of lipids from the sap-TCA

precipitate had no effect upon the amount of P which could be subsequently removed from the material insoluble at pH 9. This is described more fully in a subsequent section.

Further fractionation of pooled pH 9-*insoluble residues and protein precipitates*

In a few preliminary experiments, the residue remaining after two extractions of the sap-TCA precipitate was pooled with the protein precipitates and refractionated after a further TCA precipitation. Table 4 shows that some 70% of the P remained in the TCA supernatant. Most of the P precipitated was either not extracted at pH 9 or precipitated along with the proteins again, only 5–15% (i.e. 2–6% of the initial total) being finally obtained in nucleotide preparations.

Table 4. *Phosphorus distribution during fractionation of pooled* pH 9-*insoluble residues and protein precipitates*

(The figures refer to three separate fractionations of pooled material)

Percentage total P in:	
5% TCA supernatant	70·2, 71·4, 63·0
Nucleotide preparations	4·5, 1·9, 6·1
Percentage P of TCA precipitate in:	
pH 9-insoluble residues	38·6, 29·4, 51·6
Nucleotide preparations	14·9, 6·4, 11·5
Protein precipitates	25·2, 47·3, 22·7

Phosphorus content of pH 9-*insoluble residues*

The residue from sap-TCA precipitate after two extractions at pH 9 usually consisted of a gel which did not sediment cleanly at 8000 rev./min., and still contained 5–15% of the P of the sap-TCA precipitate. The material was difficult to mix effectively with extracting liquid, but 50–60% of its P could be removed by repeatedly washing with water (Table 5). If the material were given a preliminary treatment with 5% (w/v) TCA and then washed several times with water, a larger proportion (70–80%) was extracted. This is probably due to the change in texture of the material, on treatment with the TCA, to a hard, granular form, readily sedimenting at 1500 rev./min., for, in samples that had been washed several times with water until no more P was extracted, treatment of the residues with 5% (w/v) TCA liberated more P and in this way 70–80% of the original amount could again eventually be extracted.

Effect of incubation on the pH 9-*insoluble residues*

Increases in the amount of P that could be extracted by water from the pH 9-insoluble preparations, were observed on raising the temperature and extending the time of contact between the solvent and the sample. Lipid-free material incubated aseptically with water at 37° for 2–3 days yielded 80–90% of its P in water extracts. Samples thus treated did not liberate further P by subsequent treatment with TCA. The increased liberation is probably due to enzyme activity, as small amounts of inorganic phosphate were present in the extracts. Furthermore, when these extracts were treated with Buell's (1943) reagent the amount of P present in the precipitates increased with time to a maximum value after 2 days' incubation.

Dialysis was investigated as a means of separating nucleotides and nucleosides from undegraded ribonucleic acid and polynucleotide material following incubation of the pH 9-insoluble samples. Portions were suspended in water, adjusted to pH 7 and incubated at 37° for 24–36 hr.,

Table 5. *Liberation of phosphorus from* pH 9-*insoluble residues by further solvent action*

(Sap (1 l., 273 mg. P) was precipitated with TCA. The washed sap-TCA precipitate (41 mg. P) was split into two equal portions, one of which was extracted with lipid solvents, before extraction at pH 9. The residues insoluble at pH 9 contained 2·5 mg. and 2·36 mg. P respectively. They were suspended in water and divided into two equal portions, and washed with water alone or with 5% (w/v) TCA followed by water. The amount of P extracted by these various treatments is recorded in the table as the % of the initial total of the precipitates. In the case of the lipid-free samples, these percentages have been calculated to include the phospholipid P removed from the sap-TCA precipitate before alkaline extraction.)

Previous treatment of sap-TCA precipitate	None	Extracted with lipid solvents
Washed with water alone		
Extract:		
Lipid solvent	—	5·7
1st water	49·2	44·2
2nd water	7·2	11·2
3rd water	3·0	4·8
4th water	0·8	2·1
Total removed by water	60·2	62·8
Total P extracted	60·2	68·5
Washed with water and TCA		
Extract:		
Lipid solvent	—	5·7
1st water	49·5	41·7
5% TCA	18·7	12·5
2nd water	5·1	11·2
3rd water	4·0	11·0
Total removed by water and TCA	77·3	76·4
Total P extracted	77·3	82·1

Table 6. *Effect of incubation of* pH 9-*insoluble residues on liberation of dialysable phosphorus*

(Sap from grass (mixed *Festuca* spp.) (1 l., 619 mg. P) was precipitated with TCA. The washed sap-TCA precipitate (99·7 mg. P) was treated with lipid solvents and then extracted twice at pH 9. The residue (14·7 mg. P) was suspended in water and divided into two equal portions. One was adjusted to pH 7 and incubated at 37° for 36 hr., taken to pH 9 and dialysed against NaOH, pH 9, as described in the text. The other portion was taken to pH 9 and dialysed without previous incubation. The inorganic and total P contents of the dialysates are recorded as % of the total initial P of the residue.)

	Fractions as % of total initial P	
	Incubated at 37°	Not incubated
Inorganic P	32·4	13·0
Total P	51·6	38·3

taken to pH 9 and dialysed at room temperature against NaOH, pH 9 (1·5 vol., changed every 12 hr.), till the dialysates contained less than 1–2 μg. P/ml. Control samples were brought to pH 9 immediately and dialysed in the same way. In each case, the dialysates were examined for total and inorganic P content. Incubated samples always showed marked increases over the un-incubated controls, in both inorganic and total P contents of the dialysates. The figures in Table 6 refer to lipid-free samples. Preparations from lipid-containing sap-TCA precipitates showed the same thing.

The P contained in extracts from incubated material is not solely in the form of mononucleotides. This was shown by the fact that if a sample were incubated for 24 hr. and then dialysed at pH 9 until no further P passed through the membrane, about 90% of the P of the residual material (still at pH 9) was in solution. About half of it separated with the proteins when they were precipitated from the dialysed solutions by addition of $(NH_4)_2SO_4$ to 40% saturation.

The above results clearly indicate retention by the insoluble residues of small amounts of the enzyme activity of the original sap. This was confirmed by analysis of the purine and pyrimidine contents of the incubation extracts, and by examination of the sap-TCA precipitates for presence of enzyme activity.

did not liberate further P on incubation. The extracts obtained after boiling contained less P than did those after incubation, and the ratio of purines to pyrimidines becoming soluble was greater (Table 7). As in the case of the incubated samples, more adenine was obtained when the residues were treated at 100° for 30 min. with 5% (w/v) TCA, but even so, about 10% of the initial pH 9-insoluble P still remained insoluble after these treatments. The experiment whose results are presented in Table 7, was repeated a further four times with similar results.

It will be seen that there was no significant difference in either the total amount of P extracted or in the purine and pyrimidine composition of the combined extracts, whichever of these two series of extractions of pH 9-insoluble residues was followed. However, enzyme activity occurring during incubation at 37° leads to extracts containing pyrimidines, most of which are not liberated when enzymic activity is destroyed by boiling the samples.

Phosphorus associated with protein precipitates

The figures in Table 1 show that a large proportion of the P in the pH 9 extracts of the sap-TCA precipitates separated together with the proteins when these were precipitated from the extracts. As in the case of the pH 9-insoluble residues, this appears to exist in polynucleotide combination. If the precipitates were redissolved in NaOH, pH 9,

Table 7. *Purine and pyrimidine composition of the phosphorus fractions obtained from* pH 9-*insoluble residues by various treatments*

(Sap (1 l., 0·213 g. P) was converted to the sap-TCA precipitate (0·047 g. P). This was treated with lipid solvents and then extracted twice at pH 9. The residue (10·34 mg. P) was suspended in water and split into two equal portions. One portion was incubated at 37° for 24 hr. at pH 7. After centrifuging down, the residue was washed twice with water on the centrifuge, treated with 5% (w/v) TCA at 100° for 30 min., centrifuged down and washed with water again. The combined extracts contained 4·63 mg. P. The other portion was treated in exactly the same way except that the initial incubation was replaced by a 5 min. boiling period to destroy enzyme activity, and yielded 4·79 mg. P in the combined extracts. The purine and pyrimidine content of the extracts is given as molecules base/100 atoms P in the combined extracts.)

	Extract	Adenine	Guanine	Total pyrimidines	Phosphorus
		(molecules/100 atoms total P)			(atoms)
Incubated at 37°	Incubation	1·6	8·1	57·9	65·2
	Water (2)	0·8	2·0	3·8	6·9
	5% (w/v) TCA	6·2	3·6	11·7	21·4
	Water	1·8	1·3	4·3	6·5
	Total	10·4	15·0	77·7	100
Boiled 5 min.	Boiled	1·4	10·7	24·8	40·0
	Water (2)	1·0	—	5·3	6·6
	5% (w/v) TCA	5·7	2·3	34·4	42·7
	Water	1·4	—	8·7	10·7
	Total	9·5	13·0	73·2	100

Nature of phosphorus fractions liberated on incubation of pH 9-*insoluble residues*

Incubation of lipid-free pH 9-insoluble residues led to extracts containing predominantly pyrimidines (Table 7). The proportion of adenine in the samples, however, was much greater than in the nucleotide preparations. Further amounts of adenine were liberated when the residues after incubation were heated in 5% (w/v) TCA at 100° for 30 min. About 10% of the P present before incubation remained with the insoluble residues after these treatments.

The enzyme activity of the samples was destroyed by boiling for 5 min. After such treatment the preparations immediately after precipitation and kept at 4° for several hours before re-precipitation, only an insignificant fraction (about 5%) of this P failed to reprecipitate.

The precipitates displayed enzymic activity. Samples were incubated at 37° in water at pH 7 for 24 hr., taken to pH 9 and dialysed against NaOH, pH 9 (1·5 vol., changed every 12 hr.), at room temperature, till the dialysates contained less than 1–2 μg. P/ml. The dialysates showed marked increases in both total and inorganic P contents over those from control samples which were dialysed at pH 9 without prior incubation. The effect was shown by both lipid-containing and lipid-free samples of proteins from sap-TCA precipitates.

About 90% of the P remaining non-dialysable after incubation was soluble at pH 9. If the proteins in these extracts were thrown out by 40% saturation with $(NH_4)_2SO_4$, 50–70% of the P was co-precipitated.

Table 8. *Effect of incubation of protein precipitates upon liberation of dialysable phosphorus*

(Sap (1 l., 212 mg. P) was treated with TCA. The sap-TCA precipitate (29·6 mg. P) was split into two equal portions. One portion was fractionated at pH 9 without removal of lipids. The other was extracted with lipid solvents prior to fractionation. The protein precipitates separating from the alkaline extracts of the sap-TCA precipitates at pH 6, contained 1·04 mg. P and 1·68 mg. P respectively. Each precipitate was then split into two equal portions. One portion was, in each case, incubated at 37° in water at pH 6 for 24 hr., taken to pH 9 and dialysed as described in the text: the remaining portions were dialysed at pH 9 without previous incubation. The inorganic and total P contents of the dialysates are recorded in the table as % of the total initial P of the protein precipitates.)

	Lipids present		Lipids absent	
	Incubated	Not incubated	Incubated	Not incubated
Inorganic P	18·5	6·0	17·7	9·8
Total P	82·7	63·5	80·6	54·0

Effect of incubation of protein precipitates on nature of phosphorus fractions liberated

Extracts obtained by incubating the protein precipitates at pH 6 for 24 hr. contained an excess of purines over pyrimidines (Table 9). Further quantities of nucleotides, also containing more purines than pyrimidines, were released when the residues were heated at 100° for 30 min. in 5% (w/v) TCA. About 10% of the P initially present failed to become soluble following these treatments.

Boiling a suspension of the precipitates at pH 6 for 5 min. destroyed the enzymic activity. The boiled extracts contained rather less P than did the incubation extracts, but as was found with incubated samples, purines predominated over pyrimidines in the extracts. Treatment of the residues with 5% (w/v) TCA at 100° liberated further quantities of P, and a total of about 90% of the P initially present could be extracted from the protein precipitates by these combined treatments. Table 9 shows that approximately the same total amounts of P, of purines and of pyrimidines, are liberated, whichever set of extraction conditions are employed. However, enzymic activity during incubation of the protein precipitates gives extracts containing more nitrogen bases (especially purines) than does boiling the samples, which destroys the enzymes. Fractionation of the P of the protein precipitates by the above methods gave similar results on several more samples.

Fractionation of chloroplast-TCA precipitate

Preparation and properties of mononucleotide solutions

Chloroplast-TCA precipitate was extracted once with 95% ethanol and then with ethanol-ether (7:3, v/v) until the extracts were colourless. The lipid-free residue was then put through the same alkaline fractionation procedure as was used for the preparation of nucleotides from sap-TCA precipitates.

The amounts of P obtained in various fractions in this way, are given in Table 10. These figures refer to chloroplasts obtained from the sap preparations whose P distribution on fractionation is detailed in Table 1. The figures in these two tables differ in a number of respects. Sap-TCA precipitates contain smaller amounts of P extractable by lipid solvents (5–10% of the total) than do chloroplast-TCA precipitates. Two lipid-containing chloroplast-TCA precipitates retained only about 30% of their P after two extractions at pH 9, instead of the 40–55% remaining in samples previously treated with lipid solvents. However,

Table 9. *Purine and pyrimidine composition of the phosphorus fractions obtained from protein precipitates by various treatments*

(Sap (1 l., 96·1 mg. P) was converted to the sap-TCA precipitate (37·8 mg. P). On treatment with lipid solvents followed by fractionation, as described in the text, this yielded a protein precipitate at pH 5 containing 3·8 mg. P. This precipitate was suspended in water and split into equal portions. One portion was incubated at 37° for 24 hr. at pH 6. After centrifuging down, the residue was washed twice with water, heated in 5% (w/v) TCA at 100° for 30 min., again centrifuged down and washed twice more with water. The extracts contained a total of 0·84 mg. P. The second portion was treated in exactly the same way, except that the initial incubation was replaced by a 5 min. boiling period to destroy enzyme activity, and yielded 0·82 mg. P in the combined extracts. The purine and pyrimidine contents of the various extracts is given as molecules base/100 atoms P in the combined extracts.)

	Extract	Adenine	Guanine	Total pyrimidines	Phosphorus
		(molecules/100 atoms total P)			(atoms)
Incubated at 37°	Incubation	15·7	45·1	6·5	69·5
	Water (2)	1·2	—	—	1·3
	5% (w/v) TCA	4·9	18·4	3·3	27·1
	Water	2·1	—	—	2·1
	Total extracted	23·9	63·5	9·8	100
Boiled 5 min.	Boiled	11·9	37·5	3·4	52·2
	Water (2)	1·8	—	1·7	3·4
	5% (w/v) TCA	7·4	26·7	7·4	40·9
	Water (2)	2·2	1·1	—	3·5
	Total extracted	23·3	65·3	12·5	100

even when allowance is made for the decreased extent of extraction following removal of lipids, there is still a much smaller percentage P extraction from chloroplast-TCA precipitates than from sap-TCA precipitates. Again, only a small proportion of the P in the pH 9 extracts could be isolated in mononucleotide preparations. About half of this pH 9-soluble P is precipitated, together with the proteins, from the extracts on acidification, but its nature has not been investigated further.

Incubation of the pH 9-insoluble residues at pH 6 led to increases, over non-incubated controls, in the amount of P that could be subsequently dialysed away from the preparations at pH 9, but these increases were small.

Incubation of the pH 9-insoluble residues at 37° for 24 hr. with a sap ribonuclease preparation of the type described by Pirie (1950) (used in preference to that described in the next section because of its low dry matter and P content) in amounts more than sufficient to cause practically complete fission in 24 hr. of a yeast nucleic acid solution (pH 6) with the same total P content as the chloroplast residues, brought out only some 50–60% of the P. Most of the remainder was released when incubation was repeated twice more with fresh enzyme solution. The remaining P (approx. 8% of the initial total) was not brought out by further incubations, or by cold or hot 5% (w/v) TCA treatment.

Table 10. *Distribution of phosphorus in some fractions from chloroplasts*

(The values given are the extreme ranges obtained with several different batches of chloroplasts.)

	Percentage of total P of the		
	Chloroplasts	Chloroplast-TCA precipitate	pH 9 extracts
Soluble in lipid solvents	15–20	20–30	—
pH 9-insoluble residues	25–40	40–55	—
Mononucleotide preparations	2–8	4–10	15–30
Protein precipitates	10–15	15–20	45–55

Nucleotide preparations and protein precipitates account in most cases for about 70% of the alkali-soluble P. About half of the remainder is found in the aqueous ethanolic supernatants from the nucleotide precipitates. The nature of this baryta-soluble, ethanol-soluble P has not been investigated. Small amounts of P are also found associated with the $BaSO_4$ precipitates, and in the precipitates separating at pH 9 before addition of ethanol. It is thus usually possible to account for over 90% of the P of the chloroplast-TCA precipitate after fractionation.

The nucleotide preparations had the same general properties as did those from the sap-TCA precipitates. Analyses of typical preparations are given in Table 11, which shows that, as in the case of sap preparations, pyrimidines predominate over purines.

Table 11. *Purine and pyrimidine composition of chloroplast nucleotide preparations*

(Results of two typical experiments are presented as molecules base/100 atoms P. The chloroplasts, containing 100 mg. P in each case, gave in exp. 1 a TCA precipitate (83·1 mg. P) and nucleotide preparation (8·03 mg. P), and in exp. 2 a TCA precipitate (71·9 mg. P) and nucleotide preparation (4·03 mg. P.)

Preparation	Adenine	Guanine	Total pyrimidines
	(molecules/100 atoms P)		
1	2·1	32·1	63·6
2	0·8	34·7	61·6

Phosphorus content of pH 9-*insoluble material of chloroplast-TCA precipitate*

This is a considerable fraction of the initial chloroplast-TCA precipitate total (Table 10). If these preparations were incubated at 37° for 24 hr. in water at pH 6, 30–40% of the P was extracted. Repeated incubations at pH 6, the water being replaced every 24 hr., eventually led to extraction of about 70% of the P. Treatment of the residual material with 5% (w/v) TCA brought out a further 5% at room temperatures, and 15–20% after 30 min. at 100°.

Table 12. *Effect of incubation upon liberation of dialysable phosphorus from residues of chloroplast-TCA precipitate insoluble at* pH 9

(Washed chloroplasts were suspended in water and precipitated with TCA. The precipitate was treated several times with lipid solvents and then extracted twice at pH 9. The residue was suspended in water and split into two equal portions. One portion was adjusted to pH 6 and incubated at 37° for 36 hr., taken to pH 9 and dialysed against NaOH, pH 9, as described in the text. The remaining portion was dialysed at pH 9 without previous incubation. In exp. 1, chloroplasts (100 mg. P) gave a TCA precipitate (52·09 mg. P) and nucleotide preparation containing 2·79 mg. P. In exp. 2, the chloroplasts (100 mg. P) gave 2·78 mg. P in nucleotide preparation from a TCA precipitate containing 56·89 mg. P. The inorganic and total P contents of the dialysates are recorded as the percentage of the total initial P of the residues.)

	Incubated		Not incubated	
Experiment ...	1	2	1	2
Inorganic P	2·1	3·9	—	—
Total P	11·6	13·2	6·1	3·0

Very little P was extracted by overnight treatment of the pH 9-insoluble residues with N-$HClO_4$ at room temperatures, but incubation at 37° for 24 hr. followed by washing with water brought out over 80% of the phosphorus.

The P in extracts obtained from the pH 9-insoluble residues following incubation in water (with and without added ribonuclease), or in N-$HClO_4$, exhibited strong ab-

sorption in the region of 260 mμ. Paper chromatography of samples after hydrolysis in N-HCl for 60 min. showed the presence of adenine, guanine, cytidylic and uridylic acids. No figures have been obtained for the relative proportions of the various nitrogen bases in these extracts.

Effect of TCA precipitation upon activity of sap ribonuclease and phosphatase

Sap was split into equal halves. One lot was dialysed against distilled water at 4°, with frequent changes, for 3 days, and the volume noted. The other was precipitated with TCA and the sap-TCA precipitate, after a preliminary water wash, was suspended in water and dialysed in the same way. It was then suspended in water and made up to a known volume.

Both sap and sap-TCA precipitate preparations exhibited ribonuclease activity, with optimum fission occurring at about pH 5·5. The activity of the sap-TCA precipitate preparations was much lower than that of the sap. For example, 30% fission of a yeast nucleic acid solution was brought about by 0·125 ml. dialysed sap (equivalent to 0·107 ml. sap before dialysis) in 2·75 hr. It needed 1·5 ml. of the corresponding sap-TCA precipitate preparation (from 1·8 ml. sap before precipitation and dialysis) to give this same fission in 4·5 hr. Hence the sap-TCA precipitates still exhibited about 4% of the activity of an equivalent volume of sap.

Dialysed sap exhibited phosphatase activity towards a number of substrates. This diminished on precipitation with TCA to a much greater extent than did the ribonuclease activity. Thus, while a sample of 0·05 ml. dialysed sap (equivalent to 0·04 ml. sap before dialysis) split sodium β-glycerophosphate solution to the extent of 21% in 2 hr. at 37°, 1·5 ml. of the corresponding sap-TCA precipitate (equivalent to 1·2 ml. original sap) only caused 3% fission in 72 hr. at 37°. Hence only about 1 part in 7500 of the original phosphatase activity remained after TCA precipitation. The use of metaphosphate and yeast adenylic acid as substrates gave similar results.

DISCUSSION

Kaufmann, Gay & McDonald (1951) have shown that pancreatic ribonuclease attacks pancreatic nucleoprotein as well as the free nucleic acid. The enzyme is stable to extremes of pH, and displays marked activity at pH 9·5 (Dubos & Thompson, 1938), although it is largely inactivated in 5 min. at pH 7 and 100° (Kunitz, 1940). It would seem from the present results that the reactions leading to the formation of nucleotides from sap-TCA precipitates are at least partially enzymic in character. The results are compatible with the theory that the ribonucleoprotein in the sap-TCA precipitate is being degraded to varying extents by the sap ribonuclease. The preferential fission of pyrimidine-containing fragments from yeast nucleic acid by means of pancreatic ribonuclease has been noted by a number of workers, and has also been demonstrated for the ribonucleic acids of tobacco mosaic and turnip yellow viruses and of rabbit liver (Markham & Smith, 1951). Carter & Cohn (1950) showed that the acid-soluble digestion products produced from yeast nucleic acid by ribonuclease consisted almost exclusively of pyrimidine nucleotides, and suggested that the very small amounts of adenylic and guanylic acids also isolated were artifacts obtained during the isolation procedure from material of higher molecular weight.

The presence of purines in the nucleotide mixtures obtained here from sap may be due to purely chemical degradation of the nucleic acid (cf. Schmidt & Thannhauser, 1945). However, Pirie (1950) has already noted that sap ribonuclease differs from the pancreatic enzyme in that it brings about more or less complete fission of yeast and sap ribonucleic acids, so that the products are no longer capable of precipitation by the uranyl reagent. Moreover, Kunitz (1940) reported an optimum pH of 7·7 for pancreatic ribonuclease activity, whereas the sap enzyme has been found to exhibit maximum activity at pH 5–6. It is, therefore, possible that the presence of purines in the sap preparations is due to ribonuclease activity, and this is supported by the exhibition of enzymic activity, leading to the almost exclusive liberation of purine nucleotides, by the protein precipitates obtained during isolation of the nucleotide preparations. The low yield of nucleotides obtained when pH 9-insoluble material and protein precipitates were pooled and again fractionated also agrees with enzymic, as opposed to purely chemical, decomposition during alkaline fractionation.

Bacher & Allen (1950) recently showed that the usual methods of preparation of pancreatic ribonucleic acid yielded products containing ribonuclease. Pirie (1950) described the similar association of ribonuclease activity with tobacco sap nucleoprotein. He also showed the partial breakdown of the sap nucleic acid when prepared from the nucleoprotein by decomposition with TCA, even fresh preparations yielding a maximum of only 60% of the phosphorus in the nucleic acid. Starting with sap, he was unable to obtain any undegraded nucleic acid by precipitation with TCA, and he ascribed this partly to decomposition on prolonged contact of the nucleoprotein with TCA, and partly to enzyme action in the sap or during the preparation. The present results are in agreement with these conclusions. Enzyme activity in the sap-TCA precipitate and in various fractions obtained during the fractionation procedure, has been amply demonstrated. The evidence concerning the effect of contact with TCA on the sap-TCA precipitate, although it is not unequivocal, as the results were somewhat variable, also supports the above conclusions. Most of the present work has been done on sap-TCA precipitates collected immediately after preparation, but a small number of experiments

were performed in which these precipitates were allowed to age in contact with the TCA supernatant before being collected. In general, rather more phosphorus was found in a precipitate collected immediately after preparation than in an aged precipitate, the difference being greater the longer the precipitate was aged. It appears that sap nucleic acid is more sensitive to decomposition by TCA than are other nucleic acids (cf. Schneider, 1945). Ageing a sap-TCA precipitate decreased the solubility of the sap proteins at pH 9, but the part that went into solution retained on reprecipitation an amount of phosphorus larger than that separating with the proteins from a fresh sap-TCA precipitate. This is probably due to preferential absorption of nucleic acid or polynucleotide material by the denatured proteins.

The formation of nucleotides from chloroplast-TCA precipitates probably follows the same pattern as for sap. However, the smaller proportion of material soluble at pH 9, and of nucleotides isolable from this material, suggests either that the ribonucleic acid of chloroplasts is present in two fractions, one of which is not readily accessible to ribonuclease action, or that chloroplasts contain a ribonuclease inhibitor. Repeated additions of large amounts of sap ribonuclease were found to be necessary to extract the remaining nucleic acid from chloroplast residues, so that the low extent of extraction from the initial TCA precipitate and the slow rate of liberation of phosphorus from the pH 9-insoluble residues, do not appear to be due to enzyme inhibition. Moreover, it has recently been found (Holden, 1951) that only about 40% of the chloroplast ribonucleic acid is extracted by N-$HClO_4$ at room temperature, incubation at 37° being necessary to remove the remainder. Ogur & Rosen (1950) claimed complete extraction of ribonucleic acid from plant tissues under the former conditions.

The existence of phosphatase activity in the pH 9-insoluble residues and the protein precipitates is rather surprising in view of the low activities found for the sap-TCA precipitates. It is possible that traces of the enzyme escape denaturation during TCA precipitation, through being carried down mechanically in the precipitate, and are subsequently released when the precipitates are brought to pH 9.

It was noticed during incubation of pH 9-insoluble residues, suspensions of protein precipitates and of sap-TCA precipitates, that the suspensions rapidly settled and changed gradually, over a period of several hours, to a harder, more compact form. This did not affect the enzymic activity of the samples, since agitation did not enhance it, although it delayed completion of these changes.

SUMMARY

1. A fractionation procedure for following the breakdown of sap and chloroplast ribonucleic acids at pH 9, following precipitation with trichloroacetic acid, is described.

2. Both sap and chloroplast nucleic acids were partially degraded to a mixture of mononucleotides containing an excess of pyrimidines over purines.

3. The remaining breakdown products were found to be contained, in polynucleotide combination, principally in material insoluble at pH 9 and in protein precipitates obtained during fractionation. The purine and pyrimidine distribution following enzymic and chemical degradation of these fractions is described.

4. Differences in the ease of degradation of sap and chloroplast nucleic acids are described.

5. The extent of denaturation of sap ribonuclease and phosphatase following precipitation with trichloroacetic acid was determined.

I wish to thank the Agricultural Research Council for a grant to make this work possible.

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Amine Oxidase Activity of Rat Liver in Riboflavin Deficiency

By JOYCE HAWKINS
*Department of Pharmacology, Oxford**

(*Received* 12 *October* 1951)

The chemical constitution of the enzyme amine oxidase is not known. There are some indications that the closely related enzyme histaminase, which also catalyses the oxidation of amines, is a flavoprotein (Zeller, Stern & Wenk, 1940; Kapeller-Adler, 1949). It is known that D-amino-acid oxidase contains riboflavin in the form of flavin-adenine dinucleotide (FAD) (Warburg & Christian, 1938). In riboflavin deficiency there occurs a marked reduction in the D-amino-acid oxidase activity of rat liver (Axelrod, Soker & Elvehjem, 1939, 1940; Rossiter, 1940), and the FAD content of the liver is reduced (Ochoa & Rossiter, 1939).

In the present work the effect of riboflavin deficiency on the amine oxidase activity of rat liver has been studied. The D-amino-acid oxidase activity has been determined at the same time in order that this might serve as an index of the degree of the riboflavin deficiency obtained. In this study characteristic differences in the two enzymes have been observed.

METHODS

Diet. The method adopted for inducing riboflavin deficiency in rats was based on that described by Carpenter Harris & Kodicek (1948) who used a diet low in protein and containing succinylsulphathiazole. I am grateful to Dr E. Kodicek for his advice on the use of this diet.

The diet was composed as follows: riboflavin-free casein 10·5%, sucrose 80·35%, salt mixture (Steenbock no. 40) 5%, L-cystine 0·15%, arachis oil 3%, and succinylsulphathiazole 1%. This diet was fed to the rats *ad lib.* As supplements, they received daily: 30 μg. aneurin, 30 μg. pyridoxine, 20 mg. choline chloride, 1 μg. biotin, 250 μg. nicotinic acid and 200 μg. calcium pantothenate; three times weekly they were given 40 μg. folic acid dissolved in 1% KH_2PO_4, and once a week one drop of a 5% solution of tocopherol acetate, four drops of cod-liver oil and 1 mg. menaphthone. The animals which received this diet without added riboflavin are called deficient rats. The animals which received a daily supplement of 30 μg. riboflavin are called controls. Modifications of the diet, as used in some of the experiments, will be described later.

Animals. Litters of freshly weaned male and female piebald rats, obtained from Glaxo Laboratories, were used. They weighed about 30 g. on arrival.

About two-thirds of the rats in each litter received the deficient diet. In most litters the remaining animals were kept as controls and received daily supplements of riboflavin.

Tissue preparations. Each liver was homogenized in a small volume of 0·067 M-sodium phosphate buffer, pH 7·4. More buffer was added so that 5·0 ml. homogenate contained 1 g. tissue. About one-half of the homogenate was retained for amine oxidase determination, the remainder was centrifuged at 600*g* for 5 min. and the supernatant used for D-amino-acid oxidase determination.

Kidney extracts were prepared by first grinding the tissue with sand in a mortar, and then adding 0·067 M-sodium phosphate buffer, pH 7·4, so that 5·0 ml. suspension contained 1 g. tissue. This suspension was centrifuged for 5 min. at 600*g* and the supernatant was used for D-amino-acid oxidase determination.

Measurement of enzymic activity. All determinations were made at 37° and in O_2. Conical manometer flasks were used; the inner compartments contained filter paper and 0·3 ml. N-KOH.

Amine oxidase activity. This was determined with tyramine as substrate. The side bulbs contained either 0·4 ml. of 0·05 M-tyramine hydrochloride, or 0·4 ml. of water in the blank. The main compartment of each flask contained 1·0 ml. of rat-liver homogenate plus 0·2 ml. of 0·1 M-semicarbazide plus 0·4 ml. of water, to trap the aldehyde formed during the initial oxidation of tyramine in order to prevent further oxidation.

For the study of D-amino-acid oxidase, DL-proline was chosen, as D-proline was found to be oxidized at a high rate by the rat enzyme. The side bulb contained 0·5 ml. 1·0 M-DL-proline; the main compartment contained 0·5 ml. liver extract plus 0·5 ml. water. In the experiments with kidney the proline concentration was 0·1 M.

* Present address: Department of Chemistry and Biochemistry, St Thomas's Hospital Medical School, London, S.E. 1.

Readings were taken at 5 min. intervals, and the rate of oxidation was calculated from the O_2 uptake during the first 15 min. of the reaction. Enzymic activity is expressed in terms of q_{O_2} (tyramine) and q_{O_2} (proline) respectively, i.e. in μl. O_2/100 mg. fresh wt. tissue/hr.

EXPERIMENTS

Riboflavin deficiency and enzymic activity of liver preparations

Rats reared on the control diet showed a slow but steady growth and appeared healthy; they almost doubled their initial body weight after a fortnight. The deficient animals showed very little or no growth. Apart from diarrhoea, which became noticeable in almost all deficient animals, there were no symptoms of deficiency. The animals on the deficient diet usually survived for a fortnight, but during the third week some deaths occurred. One set of animals was maintained for a fortnight (13–15 days), and a second for 3 weeks (20–22 days). All rats in one litter were killed at one time and the enzymic activities were determined.

Administration of riboflavin and enzymic activity

It is known that on administration of riboflavin to flavin-deficient rats the D-amino-acid oxidase activity of the liver is rapidly restored (Axelrod *et al.* 1940). I have compared the effect on amine oxidase of adding riboflavin to the riboflavin-deficient diet with the effect on D-amino-acid oxidase.

In these experiments about one-third of the animals in each litter were kept as controls, one-third were maintained on the deficient diet throughout, and the remaining third were kept on the deficient diet for 11 or 12 days and then given 1 mg. riboflavin by mouth. In one series the animals received one such dose of riboflavin and were killed 24 hr. later. In a second series the rats were given 1 mg. doses of riboflavin on three successive days, and killed 24 hr. later.

The effect of riboflavin on the growth curve was immediate; the mean gain in wt. after 1 mg. of riboflavin was 4 g., and after three doses of 1 mg. riboflavin it was 7 g. The enzymic activities of the

Table 1. *Liver amine oxidase and* D-*amino-acid oxidase from control and riboflavin-deficient rats*

(Experimental details are given in the Methods section. In this and subsequent tables values are shown as means ± S.E. with the number of observations in brackets.)

Time on diet (weeks)	Diet	Amine oxidase (μl. O_2/100 mg. wet wt./hr.)	D-Amino-acid oxidase (μl. O_2/100 mg. wet wt./hr.)
2	Control	115 ±8 (23)	124·5±11·5 (18)
2	Deficient	61·5±3·5 (24)	24·5±1·5 (24)
3	Control	122 ±8 (11)	142 ±6·5 (11)
3	Deficient	54·5±5·5 (14)	18 ±3 (14)

Table 2. *The effect of riboflavin administration on the enzymic activity of liver extracts from deficient animals*

(Experimental details are given in Methods section.)

Diet	Supplement to diet	Amine oxidase (μl. O_2/100 mg. wet wt./hr.)	D-Amino-acid oxidase (μl. O_2/100 mg. wet wt./hr.)
Control	None	164 ±7·1 (9)	136±12·8 (9)
Deficient	None	62·5±3·9 (10)	25±4·4 (10)
Deficient	One dose 1 mg. riboflavin	48 ±7·5 (4)	70±21 (4)
Deficient	Three daily doses 1 mg. riboflavin	64·5±2·5 (5)	170±15 (6)

The enzymic activities of the liver from control and deficient animals are given in Table 1. After 2 weeks on the experimental diet the D-amino-acid oxidase activity in the liver extracts from deficient animals had fallen to one-fifth of that of the controls. The amine oxidase activity was reduced to 53%. When the animals were maintained on the diet for a third week the D-amino-acid oxidase activity fell to 13% and the amine oxidase activity to 45% of the controls.

Changes in the total amount of enzyme present in the liver will be described below.

liver after riboflavin administration are shown in Table 2. It can be seen that the D-amino-acid oxidase activity was increased by one dose of riboflavin, whereas the amine oxidase activity remained essentially unchanged even after three doses of riboflavin. After three doses of riboflavin the D-amino-acid oxidase activity rose to a level higher than that of the controls.

The determination of the enzymic activity per unit weight of liver does not give a full picture of the effect of riboflavin administration on the restoration of the enzymes studied, because administration of

riboflavin causes a rapid increase in liver weight. The body and the liver weights of rats on the different diets are summarized in Table 3. The mean liver weight rose from 1·66 to 2·60 g. after one dose of riboflavin, and to 3·10 g. after three doses. The total enzymic activity can be measured in μl. O_2 consumed/hr./liver; this is the q_{O_2} multiplied by the liver weight (in units of 100 mg.). The mean data thus obtained are given in Table 4 which shows that after administration of riboflavin the total amine oxidase of the liver increased. However, this increase appears slight if it is compared with the big rise in total D-amino-acid oxidase activity. Thus, after three daily doses of 1 mg. of riboflavin, amine oxidase had about doubled, whereas D-amino-acid oxidase had increased about 13-fold in these animals.

Other observers have reported that in rats on a fat-free diet low in protein and containing choline, the administration of large amounts of B vitamins resulted in the deposition of fat; this was partly prevented by inositol (McFarland & McHenry, 1948; Best, Lucas, Patterson & Ridout, 1951). Since the basic diet used in my experiments was low in protein and fat and did not include inositol, it was thought desirable to study the effect of inositol on the restoration of enzymic activity in the liver after the administration of riboflavin.

In the first series of experiments inositol was not included in the basic diet. Two litters were used; in one litter three of the deficient animals received three daily doses of 25 mg. inositol on the last 3 days of the experiment. In the second litter three animals received three daily doses of 25 mg.

Table 3. *Body weights and liver weights of rats maintained on deficient and control diets*

Diet	Time on diet (weeks)	Supplement to diet	Mean body wt. (g.)	Liver wt. (g.)	No. of obs.
Control	3	None	66	3·75±0·08	(12)
Deficient	3	None	42	1·83±0·11	(13)
Control	2	None	55	3·08±0·11	(21)
Deficient	2	None	34	1·66±0·14	(20)
Deficient	2	One dose 1 mg. riboflavin	38	2·60±0·05	(4)
Deficient	2	Three daily doses 1 mg. riboflavin	41	3·10±0·15	(6)
Control + inositol	2	None	47	2·85±0·19	(6)
Deficient + inositol	2	None	35	1·64±0·08	(11)
Deficient + inositol	2	Three daily doses 1 mg. riboflavin	45	2·68±0·13	(10)

Table 4. *Total amine oxidase and D-amino-acid oxidase activities of the liver, expressed in μl. O_2/hr./liver*

Diet	Time on diet (weeks)	Supplement to diet	Amine oxidase (μl. O_2/hr.)	D-Amino-acid oxidase (μl. O_2/hr.)
Control	3	None	4560	5350
Deficient	3	None	996	256
Control	2	None	3540	3840
Deficient	2	None	1020	405
Deficient	2	One dose 1 mg. riboflavin	1250	1820
Deficient	2	Three daily doses 1 mg. riboflavin	2000	5270
Control + inositol	2	None	3420	3690
Deficient + inositol	2	None	1130	312
Deficient + inositol	2	Three daily doses 1 mg. riboflavin	2800	5070

Inositol and the restoration of enzymic activity by riboflavin

In the course of the experiments just described, it was noticed that after administration of riboflavin, the livers of deficient rats showed an increase in fat content. This became particularly evident after centrifugation of the liver extracts, when a well-defined layer of fat formed on the surface of the extracts in the centrifuge tubes. This suggested that the rapid increase in liver weight after riboflavin administration might, at least partly, be explained by an increase of the fat content of the liver.

inositol together with 1 mg. of riboflavin. The animals were killed 24 hr. after the last dose and the enzymic activities were determined. Table 5 gives a summary of the results obtained with these litters, and for comparison, the effect of three daily doses of riboflavin alone is also given. The mean enzymic activities of the deficient animals are those from both litters; the values differ little from the mean values given in Table 1. Inositol by itself had no significant effect on either amine oxidase or D-amino-acid oxidase activity, neither did it affect the restoration of D-amino-acid oxidase after riboflavin. The combined administration of riboflavin and

inositol, however, resulted in an increase in the amine oxidase activity of the liver extracts.

In the second series of experiments inositol was included in the basic diet. Table 6 shows that both amine oxidase and D-amino-acid oxidase activities from normal and deficient rats were very much the same as in the absence of inositol. However, when three daily doses of 1 mg. of riboflavin were given to the deficient animals there occurred a rise in the q_{O_2} (tyramine) from 69 to 104·5. In the absence of inositol, the corresponding figures had been 61·5 in the deficient animals and 64·5 after the administration of riboflavin. No effect of inositol on the restoration of D-amino-acid oxidase activity was seen.

The effect of riboflavin on the total enzymic activities of the liver of rats maintained on the inositol-containing diet is shown in Table 4. On this diet, the D-amino-acid oxidase activity rose from 312 to 5070 μl. O_2/hr., about 16-fold; the amine oxidase activity rose from 1130 to 2800 μl. O_2/hr., about 2·5-fold.

D-Amino-acid oxidase activity of the kidney

In a number of experiments the D-amino-acid oxidase activity of the kidney was also determined. The mean enzymic activities found in these experiments are given in Table 7. The activity was high in the kidney extracts from control animals, and there was no significant lowering in the deficient animals. This is in agreement with the observations of Axelrod *et al.* (1940). It seems doubtful also whether the slight increases in q_{O_2} (proline) after three doses of riboflavin were significant.

Table 5. *The effect of inositol and riboflavin on the enzymic activity of liver from animals maintained on a riboflavin-deficient diet*

(Experimental details are given in the Methods section.)

Supplement to diet	Amine oxidase (μl. O_2/100 mg. wet wt./hr.)	D-Amino-acid oxidase (μl. O_2/100 mg. wet wt./hr.)
None	63·5±2·2 (5)	30± 3·4 (5)
Three daily doses 25 mg. inositol	69 ±8·5 (3)	37±10·5 (3)
Three daily doses 1 mg. riboflavin + 25 mg. inositol	88·5±3·5 (3)	163±24 (3)
Three daily doses 1 mg. riboflavin	64·5±2·5 (5)	170±15 (6)

Table 6. *Enzymic activities of liver extract from animals on a basic diet plus inositol*

(Each rat received 8 mg. inositol/day. The experiment lasted 14 days. The corresponding mean enzyme activity of liver from rats on the basic diet alone is given in square brackets.)

Diet	Supplement to diet	Amine oxidase (μl. O_2/100 mg. wet wt./hr.)	D-Amino-acid oxidase (μl. O_2/100 mg. wet wt./hr.)
Control	None	120 ±9·5 [115] (5)	129·5±19·5 [124·5] (6)
Deficient	None	69 ±6·5 [61·5] (11)	19 ±2 [24·5] (10)
Deficient	Three daily doses 1 mg. riboflavin	104·5±6·5 [64·5] (10)	189 ±16 [170] (10)

Table 7. *D-Amino-acid oxidase of rat kidney*

(Experimental details are given in the Methods section.)

Diet	Time on diet (weeks)	Supplement to diet	D-Amino-acid oxidase (μl. O_2/100 mg. wet wt./hr.)
Control	3	None	251 ±27 (5)
Deficient	3	None	243 ±12 (8)
Control	2	None	255·5±19·5 (11)
Deficient	2	None	237 ±18·5 (15)
Deficient	2	One dose 1 mg. riboflavin	230 ±46·5 (4)
Deficient	2	Three daily doses 1 mg. riboflavin	304·5±26 (6)
Deficient	2	One dose 1 mg. riboflavin + 25 mg. inositol	203 ±31·5 (3)
Deficient	2	Three daily doses 1 mg. riboflavin + 25 mg. inositol	297 ±61 (3)
Deficient	2	Three daily doses 25 mg. inositol	236 ±28 (3)

Enzymic activity on a high-protein diet

In order to find out if the lowered amine oxidase activity was due to a low protein intake, a few animals were reared on a diet which differed from the basic diet in that the casein content was raised from 10 to 20%, with a corresponding reduction in the sucrose to 70·35%. On this diet the control animals grew more rapidly than on the low-protein diet: the weights of three control animals kept for

3 weeks on the high-protein diet were 86, 89 and 89 g. as compared with a mean weight of 66 g. after 3 weeks on the low-protein diet (see Table 3). The weight of the riboflavin-deficient rats on the high-protein diet did not remain stationary; the rats put on weight during the first 2 weeks; some of the animals were maintained for as long as 5 weeks without showing any symptoms.

Three of the deficient animals were killed after 21 days and liver extracts were prepared; the mean q_{O_2} (tyramine) was 78 and the mean q_{O_2} (proline) was 19. Eight deficient and three control animals were killed after 30–38 days. Table 8 shows that the fall in both amine oxidase and D-amino-acid oxidase activities had not been prevented by increasing the protein content of the diet; the mean values for q_{O_2} are similar to those of Table 1.

Table 8. *Enzymic activity of liver extracts from animals maintained for 30–38 days on a diet containing 20 % protein*

(Experimental details are given in the Methods section.)

Diet	Amine oxidase	D-Amino-acid oxidase	Total amine oxidase	Total D-amino-acid oxidase
	(μl. O_2/100 mg. wet wt./hr.)		(μl. O_2/hr./liver)	
Control	132±13·4 (3)	64±4·1 (3)	6700	3270
Deficient	50±3·5 (8)	19±4·4 (8)	1260	466
Deficient, then 3 daily doses of 1 mg. riboflavin	86±12 (5)	134±13·6 (5)	3948	5895

Some of the animals were maintained on the high protein diet without riboflavin for 5 weeks; they then received three daily doses of 1 mg. of riboflavin before they were killed. The data for enzymic activity of the liver are also given in Table 8. As in the earlier experiments, D-amino-acid oxidase activity was restored; there was, however, also some increase in the amine oxidase activity: the mean figures for q_{O_2} (tyramine) rose from 50 to 86. Table 8 also contains the data for the total enzymic activity of the liver; these show that the amine oxidase had trebled after three doses of riboflavin. The mean liver weight of the deficient animals was 2·51 g.; it increased to 4·56 g. after riboflavin. The mean liver weight of the control animals was 5·12 g.

DISCUSSION

The experiments reported confirm that on a diet free from riboflavin which incorporates succinylsulphathiazole, rats quickly develop a state of deficiency; the rapid increase of body weight which occurs when riboflavin is added to the diet is evidence of the specific character of this condition.

D-Amino-acid oxidase of liver. After about 2 weeks on the riboflavin-deficient diet the enzymic activity dropped to about one-fifth of that of the control animals. The data given by Axelrod *et al.* (1940), although not strictly comparable with those reported in this paper, suggest that on the diets used by them the decrease in enzymic activity was less rapid. Thus it seems that the inclusion of succinylsulphathiazole in the diet reduced the time required for establishing a riboflavin deficiency, as indicated by the fall in D-amino-acid oxidase activity. That this fall was due specifically to lack of riboflavin and not to low protein content of the basic diet, is supported by the observation that a similar fall in enzymic activity occurred when the protein content of the diet was doubled.

D-Amino-acid oxidase of the kidneys. The maintenance of both FAD content and D-amino-acid oxidase activity of the kidneys in riboflavin deficiency has already been described (Rossiter, 1940; Axelrod *et al.* 1940); in my experiments also no fall in enzymic activity could be found. The riboflavin present as FAD in the D-amino-acid oxidase of the kidneys represents such a small fraction of the total flavin content of the body that a great reduction in flavin can occur while the enzymic activity of the kidneys is maintained.

Amine oxidase. The observations on this enzyme stand in marked contrast to those on D-amino-acid oxidase. The loss of amine oxidase in the flavin-deficient animal was slow, as was the rate of increase in activity after riboflavin administration. This makes it impossible to decide whether or not this enzyme contains flavin. Loss of enzymic activity occurred on the high-protein diet as well as on the low-protein diet. The possibility remains, however, that in riboflavin deficiency protein utilization is affected and that this factor is responsible for the low amine oxidase activity in the deficient animals. It seems particularly interesting that with inositol present in the diet, riboflavin was more effective in restoring amine oxidase activity. This may mean that both flavin and inositol are contained in enzyme protein, but it is also possible that amine oxidase is a flavoprotein, and that inositol is required, either for the synthesis of a prosthetic group or of new enzyme protein.

SUMMARY

1. Rats have been made deficient in riboflavin on a diet free from riboflavin and containing 1% succincylsulphathiazole and the amine oxidase and D-amino-acid oxidase of liver extracts have been determined.

2. It has been confirmed that the D-amino-acid oxidase of the liver is rapidly and severely reduced in riboflavin deficiency and that it is rapidly and fully restored upon the administration of riboflavin.

3. The amine oxidase activity of the liver is about halved in riboflavin deficiency. There is no rapid restoration of enzymic activity on giving riboflavin. If inositol is present in the diet, some restoration of amine oxidase occurs when riboflavin is administered.

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The Effect of Thyroxine and Thiouracil on the Composition of Milk

2. THE CAROTENE AND VITAMIN A CONTENT

BY R. CHANDA AND E. C. OWEN
The Hannah Dairy Research Institute, Kirkhill, Ayr

(*Received* 13 *August* 1951)

The interactions between the utilization of carotene as a provitamin A and the activity of the thyroid gland have been much studied both for animals and for man (Kunde, 1926; Lindquist, 1938; Thomson *et al.* 1939; Drill, 1943). Experiments with rats provide a basis for the connexion (long known to clinicians and quoted by Lindquist and by Drill) between carotene and the thyroid in certain types of human illnesses. Thus it has been shown in rats (Johnson & Baumann, 1947) that thyroxine accelerates repletion of hepatic stores of vitamin A from dietary carotene, whilst thiourea has the reverse effect. Thyroxine also accelerates the depletion of hepatic stores of rats deprived of dietary carotene whilst thiourea again has the reverse effect (Kelley & Day, 1948). Furthermore, Cama & Goodwin (1949) have correlated these effects with those produced by the hormone and by a goitrogen on the faecal excretion of dietary carotene in the rat.

For many years effects of thyroxine injections on the composition of milk have been studied at this Institute (Smith & Dastur, 1940; Owen, 1948; Chanda & Owen, 1949) and it was thought that the influence of thyroxine on the liver reserves of vitamin A of animals and humans might have a parallel in its effects on the concentration of vitamin A and carotene in milk. Fellenberg & Grüter (1932) and Fasold & Heidemann (1933) claimed that thyroidectomy caused carotene to appear in goat's milk, but Kon and his co-workers (1944–6) and Smith, Niedermeier & Schultz (1948) could not confirm these observations. The purpose of the present paper is to report experiments which were begun in 1948 on lactating cows and goats in which effects of thyroxine and of the goitrogen thiouracil on the composition of milk were investigated.

EXPERIMENTAL

Experiment 1

This experiment was divided into seven periods. Six lactating Ayrshire cows were used. In period 1 all were deprived of carotene and in period 2 they all received a carotene-containing diet. In period 3 all the cows were again deprived of carotene. In period 4, the diet containing carotene was fed again to all, but cows 17 and 18 were also given subcutaneously 10 mg. DL-thryoxine/day each, while cows 19 and 20 each received 20 mg. thiouracil/day by the same route. The remaining two cows served as controls. In period 5, the carotene-containing diet was continued, but the hormonal treatments were stopped. The experiment was continued into periods 6 and 7, during which the cows all received the carotene-free diet. During period 6 each cow was given the same 'hormonal' treatment as it had received

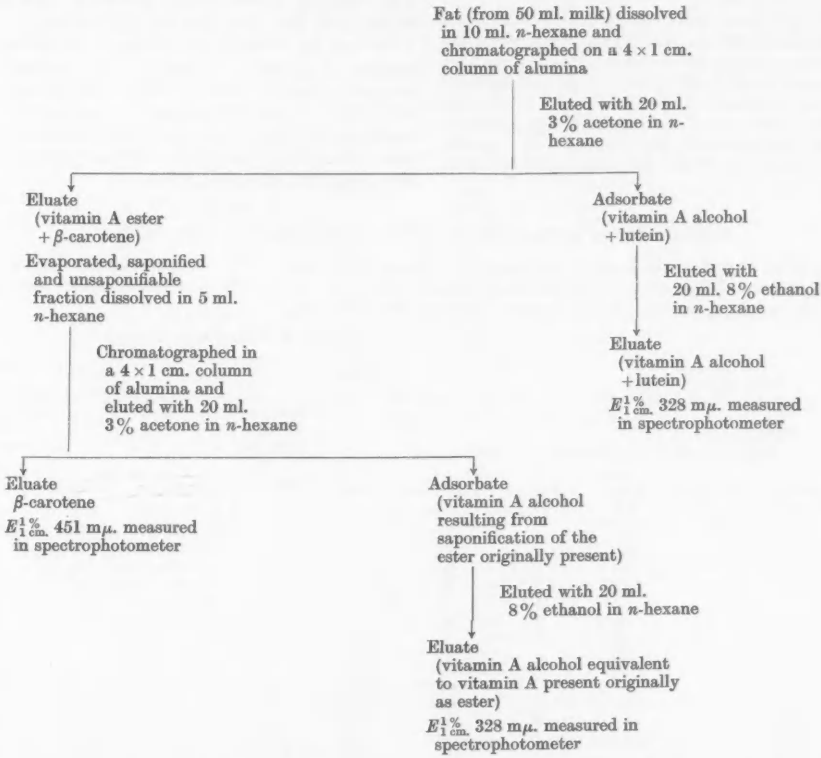
previously in period 4. In period 7 the hormonal treatments were discontinued, while all six cows were still being deprived of carotene. The duration of each period can be seen from Fig. 2.

Diets in Experiment 1. The preparation of the diets for Exp. 1 was as described by Chanda, Clapham, McNaught & Owen (1951*a*, *b*). In both carotene-containing and carotene-free diets the roughage was 10 lb. oat straw/cow/day fed in two equal portions, one in the morning and one in the afternoon.

The concentrate mixture of the carotene-containing diet consisted of 9 parts whole oats, 6 parts beans and 6 parts dried grass (all by weight).

Experiment 2

Eight lactating goats were used, one British Alpine (no. 5) and the rest British Saanens. The experiment was divided into three periods, and the hormonal treatments were confined to period 2. The goats received throughout a daily ration of 3 lb. of a concentrate mixture consisting of 9 parts by weight of oats, 6 parts of groundnut cake and 6 parts of dried grass. A carotene-free roughage was provided by oat glumes. Each goat received 1 lb. oat glumes per day in two equal portions. Water was given *ad lib.* The goats were divided into four pairs for treatment. During period 2 each goat of one pair received daily 10 mg. thyroxine, each of a second pair 20 mg. thiouracil and each of a third pair 10 mg. thyroxine, together with 1 mg. stilboestrol. The remaining pair were untreated controls. Thyroxine and thiouracil were subcutaneously injected in aqueous solution. The stilboestrol was given by the same route in solution in 1 ml. olive oil (Malpress & Owen, 1947). The dose of thyroxine proved rather too large, for the goats developed symptoms of hyperthyroidism. They rapidly lost weight, developed a wrinkling of the palpebrae and their breathing became rapid but shallow. Their appetites were adversely affected, with simultaneous polyuria. Polyuria, resulting from thyroxine treatment, has been noted before


Fat (from 50 ml. milk) dissolved in 10 ml. n-hexane and chromatographed on a 4 × 1 cm. column of alumina
Eluted with 20 ml. 3% acetone in n-hexane
Eluate (vitamin A ester + β-carotene)
Evaporated, saponified and unsaponifiable fraction dissolved in 5 ml. n-hexane
Chromatographed in a 4 × 1 cm. column of alumina and eluted with 20 ml. 3% acetone in n-hexane
Eluate β-carotene
$E^{1\%}_{1\,cm.}$ 451 mμ. measured in spectrophotometer
Adsorbate (vitamin A alcohol + lutein)
Eluted with 20 ml. 8% ethanol in n-hexane
Eluate (vitamin A alcohol + lutein)
$E^{1\%}_{1\,cm.}$ 328 mμ. measured in spectrophotometer
Adsorbate (vitamin A alcohol resulting from saponification of the ester originally present)
Eluted with 20 ml. 8% ethanol in n-hexane
Eluate (vitamin A alcohol equivalent to vitamin A present originally as ester)
$E^{1\%}_{1\,cm.}$ 328 mμ. measured in spectrophotometer

The carotene-free diet was designed to have the same content of digestible nutrients and digestible protein as the carotene-containing diet. Accordingly it contained, instead of dried grass, a mixture of whole blood, dried at low temperature (W. and J. Dunlop, Dumfries) 0·90 parts, ground straw 2·55 parts, and dry potato starch 2·55 parts (all by weight), making in all 6 parts to replace the 6 parts of dried grass in the other diet. This mixture, together with oats (9 parts) and beans (6 parts), formed the concentrate for the carotene-free rations. Each cow received 21 lb. of mixture each day in three equal portions, one in the morning, another at mid-day and the third in the evening.

in cows by Owen (1948). The dose of thyroxine was, however, based on the experiments of Meites & Turner (1948). Possibly the humidity of the Scottish summer made it more difficult for our goats to dispose of the heat burden which thyroxine treatment necessarily causes. In continental America, where Meites & Turner worked, the average humidity was perhaps not so high.

On alternate days fat and vitamin A were estimated in goat's milk. Cow's milk from alternate evening milkings was analysed for vitamin A and carotene. The goats' milk was expressed by hand, and that of the cows was taken from the recorder vessel.

Chemical methods

Separation of carotene, lutein, vitamin A alcohol and vitamin A ester present in milk fat. Milk fat was extracted by the method of Olson, Hegstedt & Peterson (1939). The carotene, lutein, vitamin A alcohol and vitamin A ester were separated as described by Chanda, Owen & Cramond (1951). The method was based on that of Ganguly, Kon & Thompson (1947), and is summarized in the scheme shown on p. 405, which gives an outline of the chromatographic treatment of the fat.

Optical measurements were made in a Unicam spectrophotometer. Total vitamin A was also determined as vitamin A alcohol in a separate sample of fat after saponification and chromatography. All the spectrophotometric readings were corrected for irrelevant absorption by the three-point method of Morton & Stubbs (1946), as previously reported by Chanda, Owen & Cramond (1951). By this means Glover, Goodwin & Morton (1948) showed that it is possible to determine vitamin A in the presence of a preponderant irrelevant absorption.

Analysis of liver and kidney. The procedure of Davies (1933) was used to extract vitamin A and carotenoids from liver and to extract vitamin A from kidneys. These extracts were then chromatographed by the procedure used for milk.

RESULTS

Differences between the compositions of cow's and goat's milk

The reliability of the scheme of analysis outlined on p. 405 was tested by measuring the recoveries of vitamin A alcohol and ester which had been added to milk fat. Duplicate samples of milk fat were dissolved in *n*-hexane, and measured amounts of pure vitamin A were added to one of each pair of samples of fat. To some samples vitamin A was added as the alcohol, to others as the ester. The results of spectrophotometric measurements are shown in Table 1 from which it will be seen that with 30 i.u. vitamin A, as ester or as alcohol, 93 % could be recovered when added to milk fat of either the cow or the goat. Even with as little as 5 i.u., 80 % could be recovered (Table 1).

Table 1. *The recovery of different forms of vitamin A added to milk fat*

(Two lots of fat were extracted from each milk sample and taken up in *n*-hexane. Pure vitamin A in *n*-hexane was added to this solution prior to chromatography in one lot; the other was kept as a control. All spectrophotometric readings were corrected for irrelevant absorption by the method of Morton & Stubbs (1946).)

Source of milk fat	Vitamin A in original milk fat from 100 ml. milk (i.u.)		Vitamin A added (i.u.) in the form of		Vitamin A in fortified milk fat (i.u.)					
					Ester			Alcohol		
	Ester (*a*)	Alcohol (*b*)	Ester (*c*)	Alcohol (*d*)	Expected (*a*+*c*)	Found (*e*)	Recovery of added vitamin (%) $\frac{100(e-a)}{c}$	Expected (*b*+*d*)	Found (*f*)	Recovery of added vitamin (%) $\frac{100(f-b)}{d}$
Cow 1	71	6	50	—	121	120	98	6	5	—
2	67	4	30	—	97	95	93	4	5	—
3	99	7	—	10	99	96	—	17	15	80
4	88	5	—	20	88	92	—	25	23	90
5	77	5	—	30	77	80	—	35	34	97
6	84	4	—	50	84	81	—	54	52	96
Goat 1	184	—	30	—	214	212	93	—	—	—
2	122	—	50	—	172	170	96	—	—	—
3	106	—	—	5	106	105	—	5	4	80
4	132	—	—	10	132	129	—	10	8	80
5	115	—	—	20	115	118	—	20	18	90
6	155	—	—	30	155	159	—	30	29	97
7	165	—	—	40	165	161	—	40	38	95
8	141	—	—	50	141	138	—	50	47	94

The method was applied to cow's and goat's milks with the results shown in Tables 2 and 3 in which milk yields and percentages of fat are also recorded. The cow's milk contained appreciable amounts of carotene (Table 2), but no carotene could be found in the goat's milk. Cow's milk contained about 6 % of its vitamin A as alcohol, but in goat's milk no measurable amount of vitamin A alcohol was present. Goat's milk contained more vitamin A per g. fat (39 i.u.) than cow's milk (21 i.u.).

Table 2. *Carotene and vitamin A in cow's milk*

(6–8 weeks post partum.)

	Cow no.							
	1	2	3	4	5	6	Mean	c.v.*
Milk yield (kg./2 days)	30·2	28·6	28·8	28·9	32·0	29·7	29·7	4·3
Fat in milk (%)	4·1	4·4	3·9	4·2	4·7	3·9	4·2	7·4
β-Carotene (μg./100 ml.)	18·5	12·1	19·5	17·6	19·4	16·5	17·3	16·0
Total vitamin A (*a*) (i.u./100 ml.)	79·5	71·4	83·8	89·7	107·9	93·5	87·6	10·2
Vitamin A ester as % of (*a*)	89·4	93·7	91·9	93·5	92·5	94·0	92·5	1·9
Vitamin A alcohol as % of (*a*)	7·5	4·7	5·9	4·6	6·5	5·4	5·8	19·2
β-Carotene (μg./g. fat)	4·5	2·8	5·0	4·2	4·1	4·2	4·1	18·2
Vitamin A (i.u./g. fat)	19·4	16·2	21·5	21·4	23·0	24·0	20·9	13·3

* c.v. (coefficient of variation) $= \frac{\text{standard deviation}}{\text{mean}} 100.$

Table 3. *Vitamin A in goat's milk*

(6–8 weeks post partum.)

Goat no.	Milk yield (ml./2 days)	Fat (%)	Vitamin A (i.u./100 ml.)	Vitamin A (i.u./g. fat)	Ester* (%)
1	2670	3·1	174	56·0	98·5
2	1940	4·1	120	29·2	97·9
3	1330	3·8	128	33·7	98·6
4	2270	3·0	142	47·2	98·1
5	4600	4·3	165	38·3	99·2
6	1780	4·2	151	35·9	97·8
7	2730	4·0	110	27·5	98·9
8	3760	3·4	150	44·2	98·2
Mean	**2635**	**3·7**	**142**	**39·0**	**98·4**
c.v.	41·0	13·6	15·4	24·7	0·5

* The figures in this column show the proportion of the total vitamin A which was present as ester, and indicate that no measurable amount of vitamin A was present in the goat's milk.

Table 4. *Vitamin A in liver and kidneys and β-carotene in liver and colostrum of goats*

Goat no.	Cause of death and date	Thyroxine (mg./day)	Liver: Vitamin A (i.u./g.)	Liver: β-Carotene (μg./g)	Kidney vitamin A (i.u./g.)	β-Carotene in the first colostrum (μg./100 ml.)
4	Hyperthyroidism (1950)	10	369	0·34	38	28
8	Hyperthyroidism* (1950)	10	253	0·00	9	12
9	Unknown (1950)	None	471	0·67	81	35
10	Bloat (1950)	None	452	0·09	77	19
5	† (1951)	3	176	0·84	8	46
7	† (1951)	3	272	Trace	33	24

* This animal received also 1 mg. stilboestrol per day.
† Killed after treatment with 3 mg. DL-thyroxine per day.

The occurrence of β-carotene along with vitamin A in the colostrum and liver of the goat

Cow colostrum is richer in carotene than the milk which is secreted later. It was noticed in the present experiments that the first colostrum of the goats was orange-yellow in colour. The immediately following samples of the secretion were yellow and were succeeded by the normal white milk of the goat. By analogy with the cow it was thought that yellow pigment of colostrum should contain β-carotene. Chromatography followed by spectrophotometry showed that the pigment contained β-carotene. The amount in the colostrum varied from goat to goat as shown in Table 4. It was further thought that this colostral carotene probably came from the goat's liver. Accordingly, the livers of six goats were examined post-mortem for β-carotene. The results in Table 4 show that β-carotene was demonstrable in most of the livers, and that the amount in the colostrum was probably determined by the amount in the liver. In view of the facts that the livers of goats examined in Liverpool by Goodwin & Gregory (1948) did not contain carotene and that one of the livers which we examined (Table 4) also contained none, the yellow pigment of

the liver was subjected to chromatography on alumina and then measured spectrophotometrically. In Fig. 1 the absorption spectra of a sample of the pigment from goat liver and of a sample of β-carotene (British Drug Houses Ltd.), both purified by chromatography on alumina, are shown. The figure demonstrates that the maxima of absorption for both the pigment from the goat liver and pure β-carotene were 423, 451 and 475 mμ. in *n*-hexane. These maxima are concordant with those quoted by Karrer & Jucker (1950) which were also measured in *n*-hexane. They also agree closely with the values obtained in petrol solution by Morton (1942). The absorption spectrum of β-carotene isolated from goat colostrum had the same absorption maxima but has been omitted for the sake of brevity. These figures leave little doubt that the pigments of both goat liver and goat colostrum contain β-carotene.

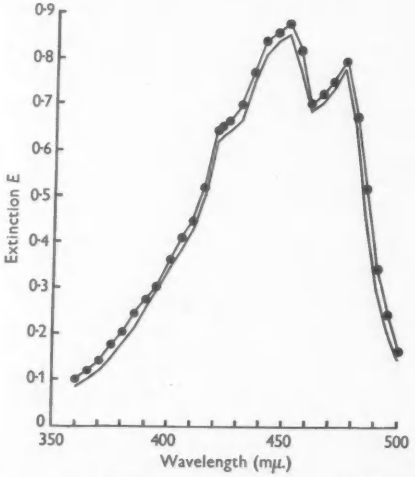


Fig. 1. Absorption spectrum of β-carotene and of the pigment isolated from goat's liver. Both pigments were dissolved in *n*-hexane after purification by chromatography on alumina. ●—●, pigment from goat's liver (goat no. 5); ——, pure β-carotene.

In both liver and colostrum the β-carotene was accompanied by another yellow pigment which from its chromatographic properties on alumina, and by analogy with cow's milk was presumed to be lutein (xanthophyll), but this latter pigment was not investigated spectrophotometrically. Our goats therefore resembled the ewes investigated by Ganguly & Deuel (1951) which also had both carotene and lutein in the liver. The kidneys differed from the liver (Table 4) in not containing carotenoids or other yellow lipochromes. The figures in Table 4 show that the livers and kidneys of the goats which had been treated with thyroxine contained less vitamin A than those of the untreated animals, nos. 9 and 10, in which the hepatic vitamin A was of the same order as that of the sheep examined by Moore & Payne (1942). The varying liver reserves listed in Table 4 will be referred to again later.

One of the goats was rather exceptional, for, within 2 days of parturition, the lactation resulting from the previous parturition had not completely ceased. The milk (which is obviously not to be confused with the preparturient colostrum which may, for experimental purposes, be expressed from the cow's udder), did not contain carotene or any other carotenoid. When this same goat came again into milk after parturition the new secretion was colostral and was yellow. The presence of β-carotene in this colostrum was also confirmed by chromatography followed by spectrophotometry.

The effect of thyroxine and thiouracil on the carotene in cow's milk (*Experiment* 1)

From Figs. 2 and 3 and Table 5 it will be seen that in period 1 the removal of carotene from the pre-experimental diet was followed by a drop in the yield of carotene in the milk and in the percentage of carotene in the milk fat of all the cows. That this drop was more precipitate than the natural decline of the yield of milk is shown by the fact that in all cases the concentration of carotene in the milk decreased after the institution of the carotene-free diet. In period 2, when carotene was replaced in the diet, the trends of the yield of carotene and percentage of carotene in the milk and the trend of carotene concentration in the milk fat were all reversed.

In period 3, carotene in the milks of all the cows decreased in much the same way as it had already done in period 1. In period 4 the two control cows behaved, with respect to carotene, much as all six cows had done in period 2, for carotene in their milk increased. In the two cows which received thyroxine in period 4 the increases in the yield of carotene per cow were noticeably larger than the increases shown by the control cows. In the two cows which were treated with thiouracil no such increases occurred at all. The carotene content of the milk fat increased more rapidly in the two thyroxine-treated cows than in the two controls. Thiouracil had the reverse effect. The effect of thyroxine in facilitating the transfer of carotene to the milk is shown by the fact that in both the thyroxine-treated cows the concentration of carotene in the milk increased at the same time as the yield of milk was increasing.

In period 5, when all the cows continued to ingest carotene, the effects of the discontinuance of the two drugs on carotene in the milk were observed. The

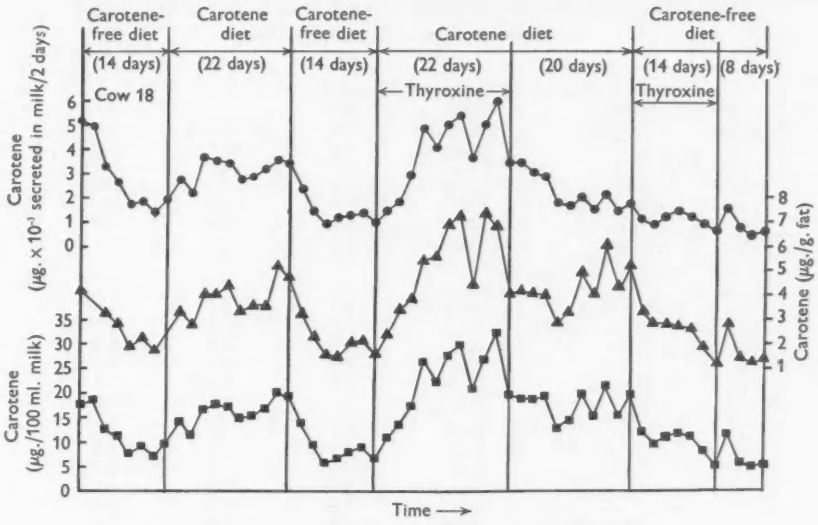


Fig. 2. The effect on the carotene of cow's milk of superimposition of treatment with thyroxine upon diets with and without carotene.

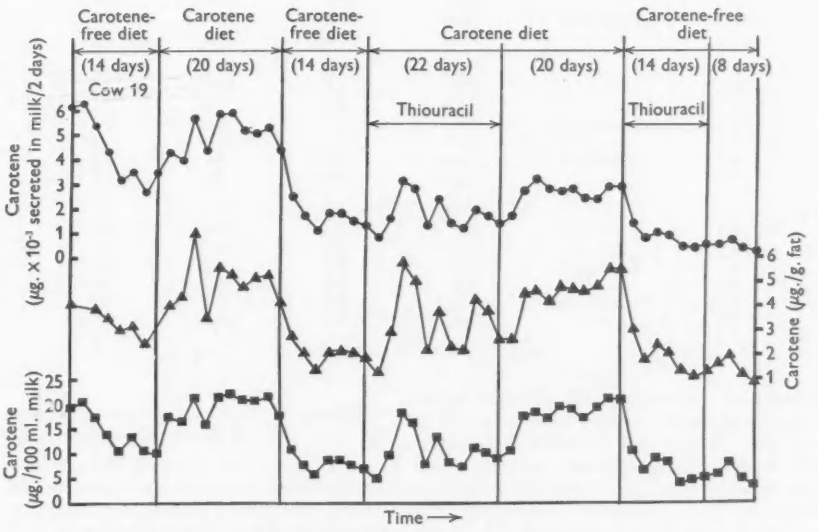


Fig. 3. The effect on the carotene of cow's milk of superimposition of treatment with thiouracil upon diets with and without carotene.

Table 5. *Effect of thyroxine and thiouracil on the mean content and the mean yield of β-carotene in the milk of cows both with and without β-carotene in the diet. Experiment* 1

(For details of dietary regimen see p. 404.)

Cow no.	Period	Treatment in periods 4 and 6	β-Carotene intake (mg./2 days)	Fat in milk (%)	β-Carotene in the milk: μg./100 ml.	β-Carotene in the milk: μg./g. fat	β-Carotene in the milk: μg. × 10^{-3} secreted/2 days
15	1	—	Trace*	4·2	15·1	3·6	4·2
	2	—	978	3·9	17·5	4·5	4·8
	3	—	Trace	3·6	9·3	2·6	2·3
	4	None	978	3·9	15·0	3·8	3·4
	5	—	948	3·8	16·5	4·3	4·1
	6	None	Trace	3·7	11·1	3·0	2·3
	7	—	Trace	3·6	2·9	0·8	0·6
16	1	—	Trace	4·1	9·0	2·2	2·4
	2	—	948	3·6	14·8	4·1	3·9
	3	—	Trace	3·7	10·1	2·7	2·5
	4	None	948	3·8	12·8	3·4	3·5
	5	—	972	4·0	15·7	3·9	3·5
	6	None	Trace	3·8	6·6	1·7	1·4
	7	—	Trace	4·0	4·2	1·1	0·8
17	1	—	Trace	3·9	12·7	3·3	3·6
	2	—	912	3·8	17·4	4·6	4·0
	3	—	Trace	3·5	7·7	2·2	1·5
	4	Thyroxine	960	4·2	23·6	5·6	5·4
	5	—	960	4·4	18·8	4·3	3·6
	6	Thyroxine	Trace	4·4	8·9	2·0	1·5
	7	—	Trace	4·3	3·6	0·8	0·8
18	1	—	Trace	4·0	11·8	3·0	2·9
	2	—	966	4·2	16·2	3·9	3·2
	3	—	Trace	4·1	8·2	2·0	1·3
	4	Thyroxine	984	4·3	22·5	5·2	4·0
	5	—	954	4·2	17·5	4·2	2·2
	6	Thyroxine	Trace	4·0	9·7	2·4	1·0
	7	—	Trace	4·1	7·2	1·8	0·9
19	1	—	Trace	4·2	15·1	3·3	4·6
	2	—	972	4·1	18·8	4·6	4·9
	3	—	Trace	3·8	7·9	2·1	1·7
	4	Thiouracil	984	3·4	10·6	3·1	1·9
	5	—	930	3·9	17·0	4·4	2·6
	6	Thiouracil	Trace	3·6	6·7	1·9	0·8
	7	—	Trace	3·9	6·1	1·6	0·5
20	1	—	Trace	4·2	12·1	2·9	3·4
	2	—	930	3·8	15·4	4·1	4·0
	3	—	Trace	3·6	8·7	2·4	2·0
	4	Thiouracil	978	3·9	8·2	2·1	1·5
	5	—	972	3·4	14·1	4·1	2·4
	6	Thiouracil	Trace	3·3	9·5	2·9	1·5
	7	—	Trace	3·5	3·5	1·0	0·5

* Approx. 2 mg./2 days from the straw.

concentration of carotene in the milk was maintained in each of the two control animals, whilst in the two which had received thyroxine it showed a marked diminution. By contrast, the two which had received thiouracil showed increases in carotene yield and in the percentage of carotene in both milk and fat. As was expected, neither thyroxine nor thiouracil appeared to affect the carotene content of the milk produced during periods 6 and 7 when the cows were receiving no carotene in their diet.

Effect of thyroxine and thiouracil on vitamin A in cow's milk (Experiment 1)

In all six cows, replacing the pre-experimental diet by one not containing carotene was followed by a fall in the yield of vitamin A in the milk, and this fall was greater than the natural decline of the milk yield because the concentration of vitamin A in the milk was also decreased (Figs. 4, 5 and Table 6). The vitamin A per g. of fat was likewise decreased in all six cows. All these three effects were reversed when

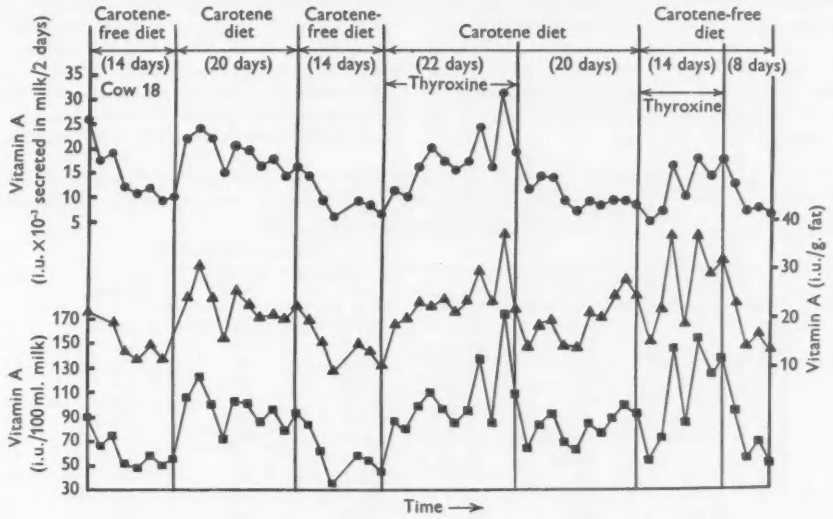


Fig. 4. The effect on the vitamin A of cow's milk of superimposition of treatment with thyroxine upon diets with and without carotene.

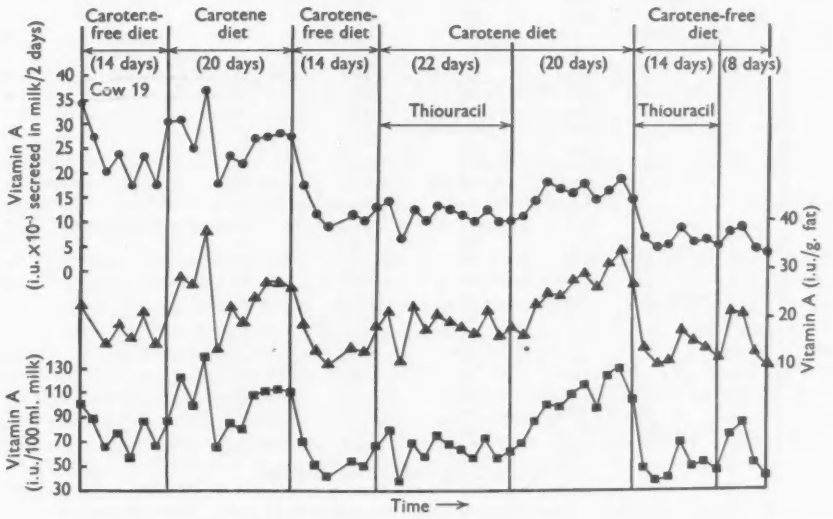


Fig. 5. The effect on the vitamin A of cow's milk of superimposition of treatment with thiouracil upon diets with and without carotene.

the cows returned in period 2 to a diet containing carotene. The increases in the vitamin A per g. fat and per 100 ml. milk were very marked in period 2. In period 3, when carotene was again withheld, a repetition of the results obtained in period 1 was observed, except that some of the cows reverted to figures for milk vitamin A which were less than those of period 1.

vitamin A in the milk of the two control cows were much enhanced by reversion to the carotene-containing diet in period 4. These increases were magnified in cows treated with thyroxine and nullified in cows treated with thiouracil.

The removal of thyroxine while the cows were still ingesting carotene (period 5) caused a sudden fall of vitamin A in both milk and fat and a sudden fall of

Table 6. *Effect of thyroxine and thiouracil on the mean content and the mean yield of total vitamin A and on the partition of vitamin A in the milk of cows both with and without β-carotene in the diet. Experiment* 1

(For details of dietary regimen see p. 404.)

Cow no.	Period	Treatment in periods 4 and 6	β-Carotene intake (mg./2 days)	Vitamin A in the milk: i.u./100 ml.	i.u./g. fat	Ester (%)	Alcohol (%)	i.u. × 10^{-3} secreted/2 days
15	1	—	Trace*	64	15·3	89	9·3	18·3
	2	—	978	90	26·3	91	6·8	24·7
	3	—	Trace	60	16·8	90	9·2	14·8
	4	None	978	72	18·7	93	7·2	16·4
	5	—	948	70	18·6	91	6·1	15·8
	6	None	Trace	52	14·1	89	8·2	10·9
	7	—	Trace	52	14·4	89	11·3	10·5
16	1	—	Trace	59	13·8	91	6·3	15·8
	2	—	948	77	21·7	94	4·4	20·2
	3	—	Trace	51	13·4	89	8·0	12·5
	4	None	948	70	18·4	93	4·7	16·0
	5	—	972	70	17·5	94	4·0	15·7
	6	None	Trace	45	11·8	89	8·6	9·6
	7	—	Trace	48	12·1	87	10·6	11·4
17	1	—	Trace	69	17·1	90	7·1	19·3
	2	—	912	88	23·2	93	5·5	20·7
	3	—	Trace	54	15·2	91	8·3	10·8
	4	Thyroxine	960	103	24·2	93	5·5	21·9
	5	—	960	80	18·6	94	4·9	15·2
	6	Thyroxine	Trace	95	21·2	83	15·1	26·3
	7	—	Trace	53	12·2	87	11·2	10·8
18	1	—	Trace	62	15·4	91	6·4	15·3
	2	—	966	95	22·6	94	4·4	18·4
	3	—	Trace	55	13·5	90	7·8	8·7
	4	Thryoxine	984	104	24·1	94	4·4	17·8
	5	—	954	83	20·0	94	3·9	10·5
	6	Thyroxine	Trace	110	27·2	80	18·3	12·4
	7	—	Trace	73	18·0	88	9·1	9·0
19	1	—	Trace	79	18·1	91	7·4	23·6
	2	—	972	102	25·5	91	6·3	27·0
	3	—	Trace	55	14·5	91	7·7	12·0
	4	Thiouracil	984	62	18·3	89	8·6	11·1
	5	—	930	98	25·4	92	6·2	15·0
	6	Thiouracil	Trace	49	13·4	90	7·3	5·8
	7	—	Trace	70	18·4	88	8·1	6·6
20	1	—	Trace	66	15·5	92	7·4	18·5
	2	—	930	95	27·6	92	5·5	24·2
	3	—	Trace	53	14·4	90	7·2	11·9
	4	Thiouracil	978	61	15·9	89	8·0	11·4
	5	—	972	92	27·7	93	5·1	16·0
	6	Thiouracil	Trace	45	13·7	87	8·2	7·0
	7	—	Trace	36	10·2	89	8·9	5·0

* Approx. 2 mg./2 days from the straw.

In period 4 the effects of the hormonal treatments were evident. Thyroxine caused a very marked rise in the vitamin A per g. fat and per 100 ml. milk of both the cows which were treated with it, while the behaviour of the two cows which received thiouracil resembled that of the two controls. The yields of the vitamin A yield. Discontinuance of thiouracil caused effects of the reverse kind which were, however, not quite so marked (Fig. 5).

An interesting feature of Tables 5 and 6 is the effect, on the concentration of carotene and vitamin A in the fat, of discontinuance of thiouracil while

carotene was being ingested. This effect was strikingly similar to that of giving thyroxine. Conversely, it is true to say that the effect of discontinuing thyroxine in cows nos. 17 and 18 was similar to that of giving thiouracil to cows nos. 19 and 20.

Partition of vitamin A between alcoholic and ester forms in cow's milk

Fig. 6 and Table 6 show that when, during periods 1, 3, 6 and 7 the cows were deprived of carotene, the proportion of vitamin A present in the milk in the alcohol form increased. When carotene was being ingested, reversion to the initial percentage occurred. During period 6, the two cows which received thyroxine simultaneously with a carotene-free diet exhibited very large increases in the proportion of vitamin A present in the form of alcohol in the milk. These increases were spasmodic and coincided with increases of the total concentration of vitamin A in the milk; in these cases most of the increase of the total vitamin A was due to the increase of the alcohol fraction.

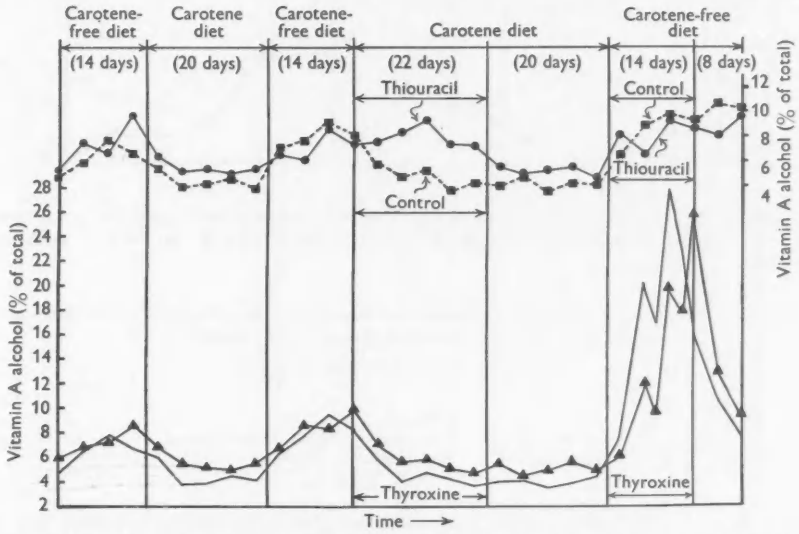


Fig. 6. The effect on the vitamin A alcohol of cow's milk of superimposition of thyroxine treatment (lower two curves) upon diets with and without carotene. Of the two upper curves, one shows the effect of dietary treatment alone and the other shows the lack of effect of superimposition of thiouracil treatment upon each diet. ●—●, cow no. 20; ■- - -■, cow no. 16; ▲—▲, cow no. 17; ——, cow no. 18.

Effects of thyroxine, thiouracil and stilboestrol on the vitamin A in goat's milk (Experiment 2)

Numerous analyses of milk from many goats confirmed the well known observation that carotenoids occur only in minute traces in goat's milk. The vitamin A in goat's milk was found to be all in the esterified form, so that free vitamin A was absent. For this reason, therefore, and also because the goats were never deprived of carotene, no attempt was made to partition vitamin A in this experiment. In general the effects were similar to those already reported for total vitamin A in cow's milk. Thus thyroxine caused an increase in the vitamin A both per 100 ml. milk and per g. fat, even although treatment with thyroxine caused an increase of the percentage of fat in the milk (Fig. 7 and Table 7). In goat no. 3, which received thyroxine alone, these increases were accompanied by a transient increase in the milk yield. In each goat which received thyroxine, whether with or without stilboestrol, there were marked increases in the vitamin A per 100 ml. milk and in the vitamin A per g. fat. Of two goats, nos. 4 and 8, which died of hyperthyroidism, one (no. 8) had received stilboestrol as well as thyroxine. In each of these two animals the secretion of the watery phase of the milk regressed much faster than the secretion of fat, so that average fat percentages for period 2 were 8·3 and 9·9 compared with 4·8 and 4·4 in the other two goats which received thyroxine. In goat no. 7 stilboestrol administration caused lactation to cease at the end of period 2 (Fig. 8), but a week later (6 days after the discontinuance of the stilboestrol) she came back into milk at the same yield as

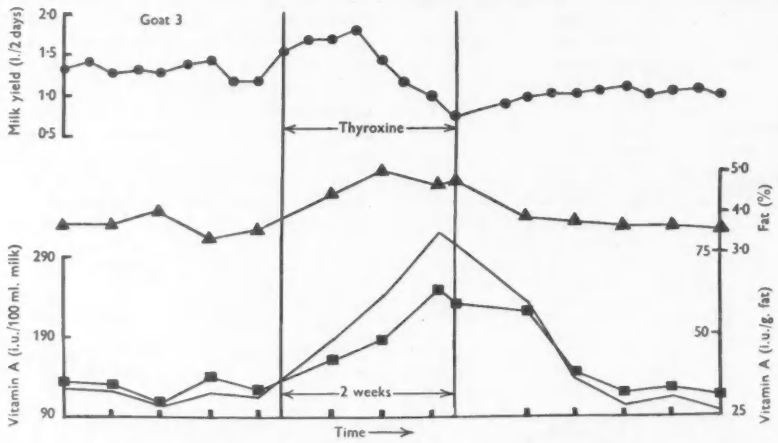


Fig. 7. Milk yield, percentage fat in the milk and the vitamin A content of milk and fat of a thyroxine-treated goat. ——, vitamin A (i.u./100 ml. milk); ■—■, vitamin A (i.u./g. fat); ▲—▲, fat (%); ●—●, milk yield (l./2 days).

Table 7. *The effect of thyroxine, thiouracil and thyroxine plus stilboestrol on the mean fat and the mean vitamin A contents of goat's milk. Experiment* 2

(For details of dietary regimen see p. 405.)

Goat no.	Period	Treatment in period 2	Dietary carotene (mg./2 days)	Fat (%)	Vitamin A: i.u./100 ml. milk	Vitamin A: i.u./g. fat	Vitamin A: i.u. $\times 10^{-3}$ secreted/2 days
1	1	—	139	3·1	179	57	4·4
	2	None	124	3·3	172	53	3·9
	3	—	100	3·3	168	52	2·7
2	1	—	94	4·3	114	27	1·8
	2	None	82	4·6	107	23	1·2
	3	—	45	4·2	97	23	2·2
5	1	—	143	4·1	163	40	7·3
	2	Thiouracil	133	3·2	104	33	4·5
	3	—	123	3·6	108	30	4·1
6	1	—	138	4·3	156	36	2·6
	2	Thiouracil	109	3·8	107	30	1·5
	3	—	93	4·1	120	29	1·0
3	1	—	122	3·7	119	32	1·6
	2	Thyroxine	70	4·8	261	55	3·5
	3	—	109	3·7	136	36	1·3
4	1	—	117	3·1	134	43	2·8
	2	Thyroxine	40	4·4	225	51	1·3
	3	—	—	—	—	—	—
7	1	—	142	3·9	112	29	3·4
	2	Thyroxine + stilboestrol	87	8·3	285	34	5·5
	3	—	111	7·6	238	30	1·0
8	1	—	114	3·4	138	41	3·1
	2	Thyroxine + stilboestrol	12	9·9	441	45	2·2
	3	—	—	—	—	—	—

previously. In this goat, as she rapidly dried off, both the percentage of fat in the milk and the percentage of vitamin A in the milk increased rapidly. When she resumed lactation both the fat and vitamin A were of about the same magnitude as they had been when lactation ceased (Fig. 8). Neither when she ceased nor when she resumed milking did this goat show any evidence of carotenoids in the milk.

supposed, from the work of Hoch & Hoch (1946), to be in the alcohol form. However, Chanda (1951) has found that cows at grass have some 16–30 % of their blood plasma vitamin A in the ester form. Glover, Goodwin & Morton (1947) have adduced evidence that reserves of vitamin A are present in the liver as the ester but are in equilibrium with vitamin A alcohol in the blood. On their hypothesis, if a cow is deprived of carotene and thereby forced to mobilize

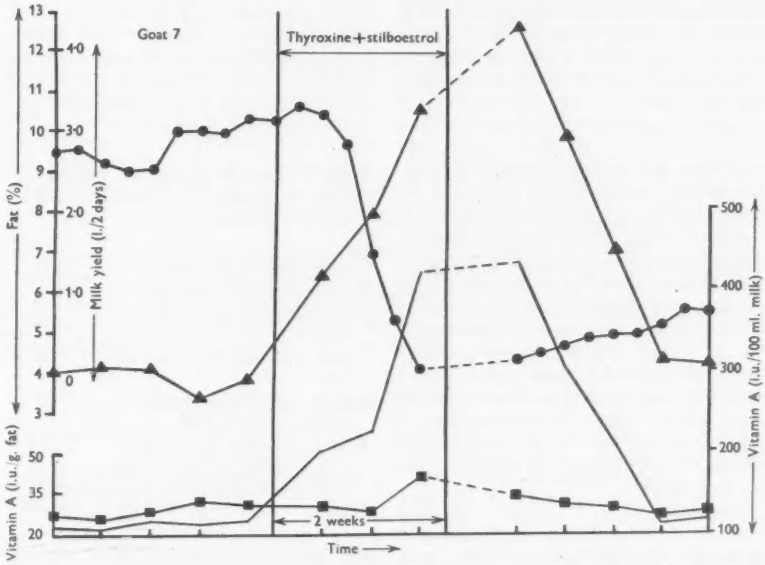


Fig. 8. Milk yield, percentage fat in the milk and the vitamin A content of milk and fat of a goat treated with thyroxine and stilboestrol. The breaks in the curves indicate that the treatment completely inhibited lactation just before the end of period 2 but that it was resumed in period 3. ——, vitamin A (i.u./100 ml. milk); ■—■, vitamin A (i.u./g. fat); ▲—▲, fat (%); ●—●, milk yield (l./2 days).

Thiouracil depressed the milk yield slightly, but at the same time it noticeably reduced the percentage of fat in the milk. This drug also depressed the amount of vitamin A per g. fat so that the yield of vitamin A in the milk was depressed (Table 7).

DISCUSSION

The results of the present experiments may be explained by supposing that when dietary carotene is absorbed it is transformed into the ester form of vitamin A (Thompson *et al.* 1950) and passes via the lymph, the thoracic duct and the blood stream to the mammary gland. In the cow some carotene probably accompanies the vitamin A ester, since both will be constituents of the chylomicrons. In the goat the accompanying carotene will be negligible. Most of the vitamin A in the blood is usually

its hepatic reserves, an increase in vitamin A alcohol in the milk would not be unexpected and this is what was found in the present experiments. Experiments by Greeves & Schmidt (1936) have shown that thyroxine accelerates the release of reserves of vitamin A from the livers of rats kept on diets deficient in the vitamin, thus explaining the effect of thyroxine in the present experiments in causing enhanced increases of vitamin A alcohol in the milk when, in period 6, the cows were deprived of carotene. The fact that vitamin A in the milk of cows was less when thiouracil was given with a carotene-free diet than it had been on the same diet without thiouracil (Table 6), can be explained on the basis of the observations of Kelley & Day (1948) who found thiouracil to retard the rate of depletion of hepatic stores of vitamin A in rats on a diet deficient in the vitamin.

Johnson & Baumann (1947) showed that, in rats, thyroxine greatly enhanced hepatic storage of vitamin A from dietary carotene but had little effect on storage from preformed dietary vitamin A. They also found that thiourea inhibited storage from dietary carotene but not from dietary vitamin A. It is therefore reasonable to suppose that the effect of thyroxine in the cow in stimulating the apparent digestibility of carotene (Chanda *et al.* 1951*b*) was due rather to the stimulation by the hormone of conversion of carotene to vitamin A in the intestinal mucosa (Glover *et al.* 1948; Thompson, Ganguly & Kon, 1949) than to any effect it may have had in increasing the absorption of carotene as such. Nevertheless, the figures in the last column of Table 5 do give reason for supposing that the increase caused by thyroxine in the output of milk carotene itself was chiefly from dietary carotene. Thus, in the control cows 15 and 16, the output of carotene in the milk was smaller in period 4 than in period 2, both of these being carotene periods. When thyroxine was given in period 4, however, cows 17 and 18 show that thyroxine caused the carotene output to be bigger in period 4 than in period 2. That this was an effect of thyroxine is shown by its reversal in cows nos. 19 and 20 which received thiouracil in period 4. That these increases in carotene output were probably of dietary rather than hepatic origin is shown by comparing periods 3 and 6 when no carotene was fed. In these circumstances thyroxine failed to cause any change in the output of milk carotene.

In this discussion it is, of course, assumed throughout that carotene, once it has passed into the lymph from the intestine, is of no further use to the cow as a source of vitamin A. It has long been known (Drummond & McWalter, 1933) that injected carotene is not converted to vitamin A by the rat and, indeed, Sexton, Mehl & Deuel (1946) have had rats dying of vitamin A deficiency while their livers contained much β-carotene resulting from injection of it. In the goat only traces of carotene could have been absorbed in these experiments (Tables 3, 4 and 7), so that the effect of thyroxine in increasing the apparent digestibility of carotene in these animals (Chanda *et al.* 1951*b*) may reasonably be attributed to an increase in the rate of conversion of carotene to vitamin A.

A considerable contribution to the vitamin A in the milk of the goats which were treated with thyroxine was probably made by their liver reserves after the overlarge dose of thyroxine had, by adversely affecting their appetites (Table 7), caused their carotene intakes to be reduced. The magnitude of this hepatic contribution can be judged by comparing, in Table 4, the liver reserves of the treated and untreated goats.

Tables 2 and 3 show that the goat's milk was richer in vitamin A than the cow's milk and that the goat's milk unlike the cow's milk did not contain either carotene or vitamin A alcohol. The greater amount of vitamin A in goat's milk can perhaps be related to the more active thyroid gland of the goat as shown in Table 8. In that table data on both goats and cows from the present experiments and from those of Chanda *et al.* (1951*a*) are presented together with the data of Schultze & Turner (1945) on the rates of secretion of thyroxine by cows and goats. The table suggests that the greater apparent digestibility of carotene by goats is associated with both a higher vitamin A content of the milk and with a greater thyroid activity. The greater vitamin A content of the goat's milk could not have been a dietary effect because, as Table 8 shows, the carotene intakes of the cows and goats before hormone treatment were practically equal when calculated in terms of body weight. The larger concentration of carotene in the milk from cows treated with thyroxine leaves the observation of Fellenberg & Grüter (1932), that thyroidectomized goats secreted yellow milk, unexplained. Indeed, in the present experiments, goats treated with either thyroxine or thiouracil showed no evidence of the presence of carotenoids in the milk. Nevertheless, β-carotene was demonstrated in goat's colostrum (Table 4). It therefore seems that there is some hormonal influence on the goat's mammary gland at parturition which causes β-carotene regularly to appear together with other carotenoids in the colostrum. The appearance of carotene in goat's colostrum could hardly have been due to the thyroid which does not attain maximum activity until the peak of lactation at which time the goat's milk is free from carotenoids. Neither do oestrogens appear to be responsible for the occurrence of carotene in goat's colostrum. Thus goat no. 7 (Fig. 8), which resumed lactation after having been 'dried off' by stilboestrol, produced milk free from

Table 8. *The rate of secretion of thyroxine, the digestibility of carotene and the secretion of vitamin A in the milk of cows and goats*

	Cows	Goats
Average body weight (kg.)	450	35
Average carotene intake (mg./day)	496	42
Carotene intake (mg./kg. body weight)	1·1	1·2
Apparent digestibility of carotene (%)*	56	63
Rate of secretion of thyroxine (mg./kg. body weight)†	22	34
Vitamin A in milk (i.u./g. fat)	21‡	39

* Data of Chanda *et al.* (1951*a*).
† Data from Schultze & Turner (1945).
‡ This value does not include the carotene.

carotenoids in the resumed lactation. Similarly, another goat which was giving some carotene-free milk 2 days before parturition gave β-carotene along with other carotenoids in the post-partum mammary secretion. If, therefore, the occurrence of β-carotene in goat's colostrum is under hormonal control it does not seem from the present experiments that the hormones concerned are either thyroxine or oestrogens.

SUMMARY

The metabolism of carotene in six lactating cows and in eight lactating goats was investigated with the following results:

1. Goat's milk contained 142 i.u. vitamin A/100 ml., all of it in the ester form. Cow's milk contained 88 i.u. vitamin A, 6% of which was in the alcohol form. Unlike the cow's milk, that of goat did not contain carotenoids, but in the colostrum and in the liver of goats β-carotene was demonstrated.

2. When the cows were deprived of carotene for a fortnight their milk yields were not affected, but the concentrations of vitamin A and carotene decreased. At the same time the proportion of vitamin A present in the alcohol form increased. Superimposing thyroxine treatment upon carotene deprivation caused even greater increases of vitamin A alcohol in the milk.

3. On re-instatement of the carotene-containing diet the concentrations of both carotene and vitamin A increased at rates which were enhanced by thyroxine and diminished by thiouracil. The effects on vitamin A and carotene of discontinuing thyroxine while the cows were still ingesting carotene were similar to those produced by injecting thiouracil. Conversely, the effects of discontinuing thiouracil were similar to those of giving thyroxine.

4. Thyroxine and thiouracil produced changes in the composition of goat's milk which were similar to those found in cow's milk.

The authors wish to thank Dr M. L. McNaught and Miss H. M. Clapham for their kind collaboration during a part of these experiments. The authors also wish to thank Miss P. McGuigan, Miss S. McLauchlan and Miss M. Millar for technical assistance. Thanks are also due to Dr W. Holmes for making cows from the Hannah Herd available for these experiments.

One of us (R.C.) is in receipt of a grant from the Agricultural Research Council for which grateful acknowledgement is made.

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The Oxidation of Myoglobin to Metmyoglobin by Oxygen

2. THE RELATION BETWEEN THE FIRST ORDER RATE CONSTANT AND THE PARTIAL PRESSURE OF OXYGEN

By P. GEORGE and C. J. STRATMANN*
Department of Colloid Science, University of Cambridge

(*Received* 20 *August* 1951)

In a previous paper (George & Stratmann, 1952) we showed that the oxidation of myoglobin to metmyoglobin by oxygen at constant oxygen pressure, pH, salt concentration and temperature was first order in unoxidized myoglobin. It thus resembles the autoxidation of haemoglobin investigated by Brooks (1931). In air the oxidation rate is 4·25 times faster than with haemoglobin.

Brooks (1935) showed that the observed first order rate constant for haemoglobin autoxidation was a function of the oxygen pressure, having a well defined maximum value at about 20 mm. In this paper the results of a similar investigation are reported for myoglobin, together with the determination of the equilibrium constant of the myoglobin-oxygen reaction, K_e

$$\mathrm{Mb} + \mathrm{O_2} \rightleftharpoons \mathrm{MbO_2}$$

under the conditions of the experiments. This value of the equilibrium constant is used in an analysis of the results based on a suggestion by George (1949) that the observed first order constant for haemoglobin was a complex function given by

$$k_{\mathrm{obs.}} = k\,\frac{K}{K+[\mathrm{O_2}]}\,\frac{[\mathrm{O_2}]}{K+[\mathrm{O_2}]}, \qquad (1)$$

where K is the reciprocal of the equilibrium constant for the formation of oxyhaemoglobin. A full comparison is made between the kinetic data for myoglobin and haemoglobin.

MATERIALS AND APPARATUS

The myoglobin used in these experiments was prepared by the method described by George & Stratmann (1952). The myoglobin solutions were heavily buffered with K_2HPO_4 and KH_2PO_4, giving a total concentration of phosphate ions of 0·6 M with a pH of 5·69 at 30°.

The flow apparatus was similar to that described by Brooks (1935). The gas mixtures were prepared from cylinders of O_2-free N_2 and either air or O_2 of 'medical' purity. The gases were passed first through cotton wool. The N_2 was passed through alkaline pyrogallol solution and the O_2 or air through alkaline permanganate solution. In order to prevent deposition of moisture in the capillaries of the flow meters the gases were further washed in half-saturated NaCl. After passing through the flow meters the gases were mixed, passed through a glass coil immersed in the thermostat to preheat it, then passed through a buffer solution like that used for the myoglobin and finally led into the reaction vessel. A similar coil and tube containing buffer solution was used to cool the gas when the flask was immersed in the ice bath employed in the preliminary preparation of the MbO_2 described below. The rate of gas flow was 6 l./hr.

Kinetic measurements

The flask used in these experiments was similar to the one used by Brooks (1935) but was boat-shaped like a Meldrum-Roughton boat but without the central partition. A solution of metmyoglobin (MetMb) with a concentration of $2{\cdot}57 \times 10^{-4}$ M was run into the flask. The optical density of the MetMb having been recorded, a small quantity of sodium dithionite ($Na_2S_2O_4$) was added and the flask gently shaken until the slight excess of $Na_2S_2O_4$ was oxidized and oxymyoglobin formed. The flask was then closed, placed in an ice bath and the gas mixture passed through the flask for 1·5–2 hr. whilst the flask was shaken slowly. A sample of the myoglobin solution was removed, diluted and the optical density measured. The usual sample removed was 1 ml. and diluted with 4 ml. of buffer solution. Details of the spectrophotometric methods of analysis used are given in a previous paper (George & Stratmann, 1952). The flask was then transferred to the thermostat at 30°. At intervals throughout the experiment samples were removed and the optical density measured. During the time taken to measure the optical density of the solutions there was no observable change due to autoxidation. The starting point of the reaction was taken as being the time when the flask was placed in the thermostat. Results were recorded as the percentage of oxymyoglobin present, taking the starting point of the reaction as 100% MbO_2. Log percentage MbO_2 was plotted against time and the first order rate constant of the reaction (k hr.$^{-1}$) was calculated from the slope of the line.

Equilibrium measurements

The oxygen dissociation curve of myoglobin was determined under the same general conditions as obtained in the kinetic experiments. The reaction flask had a side arm which was of such a size as to fit into the cell carrier of the spectrophotometer. The Pyrex glass used in its construction did not absorb appreciably in the range of wavelengths used, and since the method was a comparative one the small optical density difference between this tube and the reference cell was of no consequence.

* Present address: Department of Anatomy, University College, Gower Street, London, W.C. 1.

Two slightly different techniques were used. In the first a concentration of $5{\cdot}14 \times 10^{-5}$ M-myoglobin was used and the optical densities measured at 540 mμ. In the second, one-tenth of the above concentration was used and the optical densities measured at 420 mμ. In both methods approximately 10 ml. of metmyoglobin were placed in the flask. This was allowed to run into the side arm and the optical density

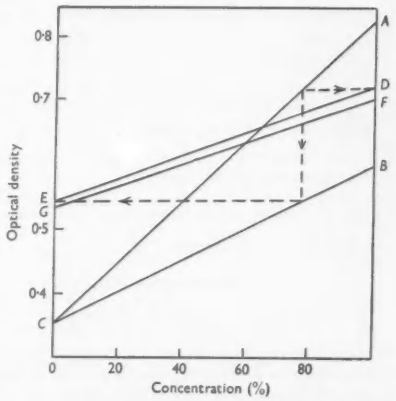

Fig. 1. Diagram used to facilitate calculation in the determination of the equilibrium constant of the reaction: $Mb + O_2 \rightleftharpoons MbO_2$ at 30° in 0·6 M-phosphate buffer of pH 5·69. The points *A*, *B* and *C* represent the optical densities (at a wavelength of 540 mμ.) of solutions containing 100% MbO_2, 100% Mb and 100% MetMb respectively. The line *AC* relates optical density to the percentage oxidation. The line *BC* relates optical density to the proportion of Mb and MetMb present in the solution if no MbO_2 is present. The point *D* gives the amount of MetMb formation which has occurred by autoxidation in the time taken for equilibration of the solution with the gas mixture. This point enables the line *DE*, which relates optical density to the proportion of Mb and MbO_2 present, after oxidation has been taken into account, to be drawn. The dotted line shows how the points *D* and *E* are derived. The measured optical density of the solution, equilibrated with the gas mixture, is used to determine the percentage of MbO_2 in the mixture by simply reading off the corresponding value on the line *DE*. The line *FG* represents a second equilibration experiment, using the same solution, when further oxidation has taken place.

was measured. The flask was removed from the spectrophotometer, placed in an ice bath and the solution run back into the body of the flask. A small quantity of $Na_2S_2O_4$ was added and the flask gently shaken until reduced myoglobin was formed. The optical density was again measured. The flask was again returned to the ice bath and shaken until all excess $Na_2S_2O_4$ was oxidized and oxymyoglobin formed. The optical density of this solution was measured. In order to facilitate calculation a diagram shown in Fig. 1 was constructed which related optical density to the percentages of MbO_2, Mb and MetMb present in the solution. *A* represents the optical density of a solution containing 100% MbO_2, while *B* and *C* represent the optical densities of solutions containing 100% Mb and MetMb respectively. Lines were drawn connecting *A* to *C* and *B* to *C*. The line *AC* relates the optical density of the solution with the proportion of MbO_2 and MetMb present in it or, in other words, the percentage oxidation. The line *BC* relates the optical density of the solution to the proportion of Mb and MetMb present in the mixture if no MbO_2 is present. A gas mixture of the desired partial pressure of O_2 was then passed through the flask which was shaken slowly in an ice bath for a period of 1 hr. The flask was then placed in the thermostat at 30° and shaken for 10–15 min. The flask was then quickly removed from the thermostat and the optical density of the solution measured. It was immediately replaced in the ice bath, opened to the air, the solution equilibrated with air and the optical density again measured. After equilibration with air the measured optical density (the point *D* on the diagram) gives the amount of oxidation of the mixture during the time taken for equilibration for the particular partial pressure of O_2, and enables the line *DE* to be drawn, which relates the optical density of the solution with the proportion of Mb and MbO_2 present, after oxidation has been taken into consideration. The dotted line on the diagram shows how this point *E* is derived. The measured optical density of the solution equilibrated with the gas mixture can now be used to determine the percentage MbO_2 in the solution by simply reading off the value which corresponds to the measured optical density on the line *DE*. The whole cycle was repeated once or twice. The line *FG* represents a second equilibration experiment, using the same solution, when further oxidation had occurred.

In the second method the technique was essentially the same. A wavelength of 420 mμ. was chosen at which MetMb and Mb are isobestic and the diagram is in consequence simplified. As the solutions absorbed to a much greater extent at this wavelength it was necessary to use a solution approximately ten times more dilute than that used in the experiments described above.

Denaturation check

The azide-metmyoglobin check for denaturation was carried out at 2 mm. O_2 pressure, it having been found previously that at 152 mm. O_2 pressure no detectable denaturation occurred (George & Stratmann, 1952).

RESULTS

Denaturation check

In 3 hr. and at a partial pressure of 2 mm. of oxygen 77·5% of metmyoglobin was formed from oxymyoglobin by autoxidation. Fig. 2 shows that some traces of denaturation of the protein are evident. This amounts, however, to no more than 1·5–1·7% of the myoglobin originally present, and it was not considered necessary to take this into consideration in the calculation of the rate constant.

Oxidation kinetics

The metmyoglobin formation was measured at 10 and 20 min. intervals and at many partial pressures of oxygen ranging from 0·3 to 760 mm. Fig. 3 shows four typical first order plots at the following oxygen pressures: 760, 1·0, 0·6 and 0·3 mm.

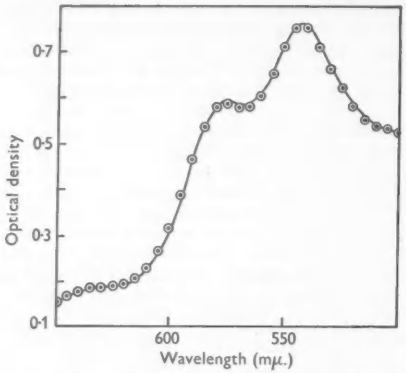

Fig. 2. Spectra of the azide-metmyoglobin complex formed before and after autoxidation of myglobin at 30° in 0·6 M-phosphate buffer of pH 5·69 at a partial pressure of oxygen of 2 mm. ——, from a solution of metmyoglobin before autoxidation; ⊙, from a solution of metmyoglobin of the same concentration after autoxidation for 3 hr.

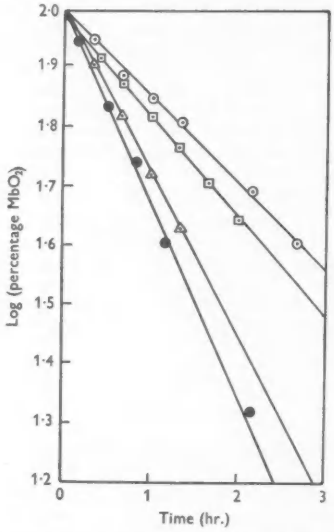

Fig. 3. Typical first order plots for the autoxidation of myoglobin to metmyoglobin in 0·6 M-phosphate buffer of pH 5·69 at 30° at four different partial pressures of oxygen. ⊙, partial pressure of oxygen of 760 mm.; ●, partial pressure of oxygen of 1·0 mm.; △, partial pressure of oxygen of 0·6 mm.; ⊡, partial pressure of oxygen of 0·3 mm.

The first order rate constants at these oxygen pressures were: 0·33, 0·75, 0·65 and 0·39 hr.$^{-1}$ respectively. Fig. 4 shows the relationship between the partial pressure of oxygen and the first order rate constant over the entire range of oxygen pressures studied. A clearly defined maximum was observed at a partial pressure of about 1 mm. oxygen. Increase of the oxygen pressure above approximately 30 mm. was found to have very little effect on the rate constant, its value at these pressures being around 0·30 hr.$^{-1}$. All these results were obtained with the same sample of myoglobin.

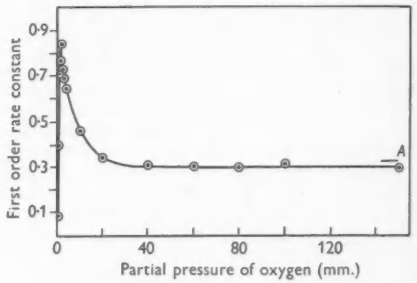

Fig. 4. The relationship between the partial pressure of oxygen and the first order rate constant for the autoxidation of myoglobin to metmyoglobin at 30° in 0·6 M-phosphate buffer, over the entire range of oxygen pressures studied. A = the first order rate constant at 760 mm. p_{O_2}.

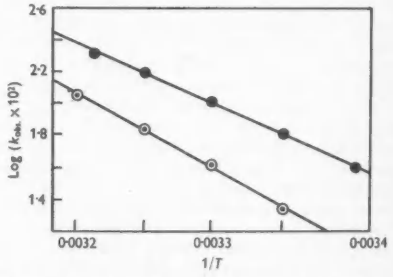

Fig. 5. Results of experiments to determine the activation energy of the autoxidation of myoglobin to metmyoglobin in 0·6 M-phosphate buffer of pH 5·69 at 4 and 760 mm. partial pressures of oxygen. ●, partial pressure of oxygen = 4 mm.; ⊙, partial pressure of oxygen = 760 mm.

The activation energy of the autoxidation

The activation energy of the reaction was determined at two partial pressures of oxygen, namely 4 and 760 mm. A range of temperatures from 22 to 38° was studied and the rate constant was determined at 4° intervals over this range. Fig. 5 shows the results of these experiments. Logarithm of the rate constant is plotted against $1/T$. Calculations from this data gives the activation energy, E, for the reaction at 760 mm. oxygen pressure as 25·0 ± 1 kg. cal., and at 4 mm. as 19 ± 1 kg.cal. The temperature

coefficient of the buffer solution used in these experiments is $-0{\cdot}03$ pH units for an increase of 10° (Cohn, 1927). The rate constant would be changed by this decrease of pH over 16° by about 10%, by analogy with Brooks's (1931) results with haemoglobin. If this is the case then the true values of the activation energies are within 1 kg.cal. of the above values.

It was found to be impossible to obtain a satisfactory value for the rate constant in an experiment carried out at 4 mm. oxygen pressure and at 38° owing to rapid denaturation of the protein at this temperature and oxygen pressure. It would appear that reduced myoglobin is more easily denatured by heat than is oxymyoglobin, since the experiment at 38° and 760 mm. oxygen pressure was reproducible and there were no detectable traces of denaturation.

The oxygen dissociation curve of myoglobin

The fraction of myoglobin in the form of oxymyoglobin at a given oxygen pressure was obtained by the method described above. Spectrophotometric analysis at 420 mμ. was used in addition to the measurements at 540 mμ. in the hope that more consistent results would be obtained. In Table 1,

Table 1. *Measurement of equilibrium constant for* $Mb + O_2$ *at 30° in 0·6 M-phosphate buffer, pH 5·69 by spectrophotometric analysis at 540 and 420 mμ.*

Wavelength (mμ.)	p_{O_2} (mm.)	Fraction MbO_2 present $(1-\alpha)$	Mean $1-\alpha$	$K_e = \frac{(1-\alpha)}{\alpha p_{O_2}}$
540	0·6	0·37 0·38	0·38	1·02
540	1·0	0·55 0·53 0·48	0·55	1·08
540	2·0	0·60 0·62	0·61	0·78
420	3·0	0·65 0·67	0·66	0·65
420	5·0	0·84 0·81 0·78	0·81	0·85
420	10·0	0·95 0·90 0·85	0·90	0·90

Mean $K_e = 0{\cdot}88 \pm 0{\cdot}12$ mm.$^{-1}$

Therefore partial pressure of oxygen for half saturation = 1·00–1·32 mm., mean **1·16**.

which records the results at a number of different oxygen pressures, it is seen that there is little to choose between the two methods. The value of the equilibrium constant K_e under the conditions of the experiments, at 30°, is $0{\cdot}88 \pm 0{\cdot}12$ from which it follows that the oxygen pressure for half saturation of the myoglobin is 1·00–1·32 mm.

ANALYSIS OF THE RESULTS

The most striking feature of the results is that myoglobin, like haemoglobin, shows a maximum oxidation rate at low oxygen pressures. In the case of haemoglobin the oxygen pressure for the maximum rate corresponds closely to that required to half saturate the haemoglobin. This also holds for myoglobin as illustrated in Fig. 6.

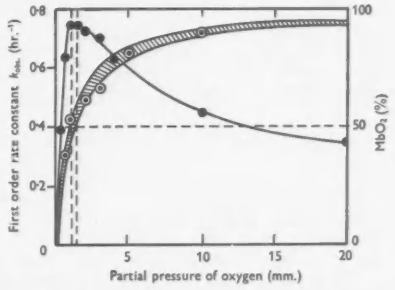

Fig. 6. The relationship between the oxygen pressure variation of the first order rate constant for the autoxidation of myglobin to metmyoglobin and the oxygen dissociation curve of myoglobin at 30° in 0·6 M-phosphate buffer of pH 5·69. ●, experimental values of the first order rate constant at partial pressures of oxygen over the range 0–20 mm. ⊙, experimental points in the determination of the oxygen dissociation curve of myglobin. The true dissociation curve lies within the shaded area, the two lines being the limits of experimental error. ----, the partial pressure of oxygen required for half saturation of the myoglobin.

George (1949) suggested that the oxygen pressure function responsible for this behaviour in the case of haemoglobin was that given in eqn. 1.

$$k_{obs.} = k \frac{K}{K+[O_2]} \frac{[O_2]}{K+[O_2]}. \qquad (1)$$

Inspection of Fig. 4 shows that this equation alone cannot adequately account for the myoglobin data. At oxygen pressures above 30 mm., $k_{obs.}$ reaches a constant value and does not continuously diminish as p_{O_2} is increased in accord with this equation. It does, however, account for the maximum value of $k_{obs.}$ at the oxygen pressure required for half saturation of the myoglobin, which suggests that the correct function for $k_{obs.}$ may be of the form:

$$k_{obs.} = k_1 \frac{K}{K+[O_2]} \frac{[O_2]}{K+[O_2]} + Y. \qquad (2)$$

The additional oxygen pressure function Y can be numerically small at low oxygen pressures and in consequence $k_{obs.}$ can be given, in effect, by Eqn. 1 and, in addition, at high oxygen pressures Y can be such that a constant value for $k_{obs.}$ is obtained. The

actual form of Y in the present data can be found in the following way. $\frac{K}{K+[O_2]}$ and $\frac{[O_2]}{K+[O_2]}$ are the values of α and $(1-\alpha)$, the fractions of uncombined myoglobin and oxymyoglobin respectively, so that eqn. 2 amounts to

$$k_{obs.} = k_1\alpha(1-\alpha) + Y. \qquad (3)$$

If k_1 can be evaluated then the variation of Y with oxygen pressure is given by $k_{obs.} - k_1\alpha(1-\alpha)$. eqn. 3 may be rewritten

$$\frac{k_{obs.}}{\alpha(1-\alpha)} = k_1 + \frac{Y}{\alpha(1-\alpha)}.$$

The problem is thus one of chosing a suitable oxygen pressure function to plot $\frac{k_{obs.}}{\alpha(1-\alpha)}$ against so that k_1 may be obtained by extrapolation. Plotting against p_{O_2} itself is not satisfactory since a definite curve is obtained. The functions $(1-\alpha)/\alpha$ and $1/\alpha$ both give plots which are reasonably linear as shown in Fig. 7, the first point lying off the line in each case. Values of k_1 obtained from the intercept are 2·62 and 2·30 hr.$^{-1}$ respectively. Table 2 gives the calculation of Y based on these two values using eqn. 3. It can be seen that the variation of Y with oxygen pressure is of the type anticipated above. The linearity shown in Fig. 7 when $\frac{k_{obs.}}{\alpha(1-\alpha)}$ is plotted against $(1-\alpha)/\alpha$ implies that Y is of the form $k_2(1-\alpha)^2$, and for the plot against $1/\alpha$ that Y is of the form $k_2(1-\alpha)$. It seems that the experimental data are not precise enough to decide between these two possibilities. This is understandable for the uncertainty in the velocity constant is about $\pm 0{\cdot}02$ hr.$^{-1}$ in each case. The slopes of the two lines are identical for their numerical value should be equal to $k_{obs.}$ at high oxygen pressures, hence $k_2 = 0{\cdot}30$ hr.$^{-1}$. For myoglobin autoxidation we may thus summarize the data in the two possible expressions:

$$k_{obs.} = 2{\cdot}62\alpha(1-\alpha) + 0{\cdot}30(1-\alpha)^2, \qquad (4a)$$

$$k_{obs.} = 2{\cdot}30\alpha(1-\alpha) + 0{\cdot}30(1-\alpha). \qquad (4b)$$

It is of interest now to examine Brooks's (1935) data on haemoglobin autoxidation to see whether there is any evidence of similar behaviour. The analysis previously referred to is given in Table 3 (George, 1949). The ratio $\frac{k_{obs.}}{\alpha(1-\alpha)}$ is not in fact constant but shows a small steady increase as p_{O_2} increases. The overall variation of this ratio, however, is far less than that obtained using Legge's

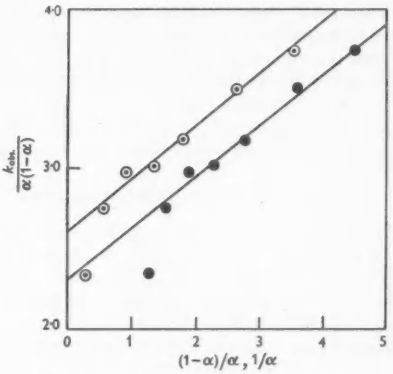

Fig. 7. Plot of $\frac{k_{obs.}}{\alpha(1-\alpha)}$ against $(1-\alpha)/\alpha$ (⊙); and $1/\alpha$ (●) in the case of myglobin.

mechanism (Legge, 1942), assuming methaemoglobin formation by the unique decomposition of $Hb_4(O_2)_2$, and for this reason analysis of the results in terms of eqn. 1 was suggested. If now $\frac{k_{obs.}}{\alpha(1-\alpha)}$ is plotted against $(1-\alpha)/\alpha$ and $1/\alpha$ as has been done in Fig. 7 for myoglobin, the two lines in Fig. 8 are obtained, thus showing that haemoglobin autoxidation follows the same pattern. In this case the two possible equations are

$$k_{obs.} = 0{\cdot}68\alpha(1-\alpha) + 4\times10^{-2}(1-\alpha)^2, \qquad (5a)$$

$$k_{obs.} = 0{\cdot}64\alpha(1-\alpha) + 4\times10^{-2}(1-\alpha). \qquad (5b)$$

Table 2. *Calculations of Y, the high pressure component of the overall first order constant for myoglobin autoxidation*

($Y = k_{obs.} - k_1\alpha(1-\alpha)$, where $k_1 = 2{\cdot}62$ or $2{\cdot}30$ hr.$^{-1}$
$\alpha = K/K+O_2$, $1-\alpha = O_2/(K+O_2)$, where $K = 1{\cdot}16$ mm.)

p_{O_2} (mm.)	$1-\alpha$	α	$\alpha(1-\alpha)$	$k_{obs.}$	$k_{obs.} - 2{\cdot}62\,\alpha(1-\alpha)$	$k_{obs.} - 2{\cdot}30\times\alpha(1-\alpha)$
0·3	0·21	0·79	0·166	0·39	(−·07)	(−0·01)
0·6	0·35	0·65	0·228	0·635	0·035	0·105
1·0	0·47	0·53	0·249	0·745	0·085	0·165
1·5	0·57	0·43	0·245	0·74	0·100	0·180
2·0	0·64	0·36	0·230	0·74	0·130	0·200
3·0	0·725	0·275	0·199	0·70	0·180	0·240
4·0	0·78	0·22	0·172	0·635	0·205	0·245
100	0·99	0·011	0·011	0·32	0·29	0·29
160	0·99	0·007	0·007	0·30	0·28	0·28
760	1·00	0·0015	0·0015	0·33	0·33	0·33

The points corresponding to the two lowest oxygen pressures do not lie on the lines, which may be attributed to uncertainty in the precise value for p_{O_2}; a small uncertainty in this region leads to large

Table 3. *Analysis of Brooks's (1935) results for haemoglobin autoxidation in 0·6 M-phosphate buffer at 30°*

(The observed first order constants are divided by the oxygen pressure function $\alpha(1-\alpha)$ based on equilibrium measurements on the haemoglobin-oxygen reaction under the same experimental conditions.)

p_{O_2}	$k_{obs.}$ (hr.$^{-1}$ × 10²)	α	$1-\alpha$	$k_{obs.}/\alpha(1-\alpha)$
4·5	9·9	0·89	0·11	1·01
6·0	12·6	0·85	0·15	0·98
13·7	15·9	0·84	0·36	0·69
16·0	17·6	0·57	0·43	0·72
25·8	17·7	0·39	0·61	0·74
33·3	14·5	0·29	0·71	0·71
63·1	10·3	0·12	0·88	0·97
92·9	8·45	0·08	0·92	1·16
122	7·40	0·06	0·94	1·30
152	6·36	0·048	0·95	1·39
435	5·17	0·035	0·965	1·52
723	4·61	0·030	0·97	1·59

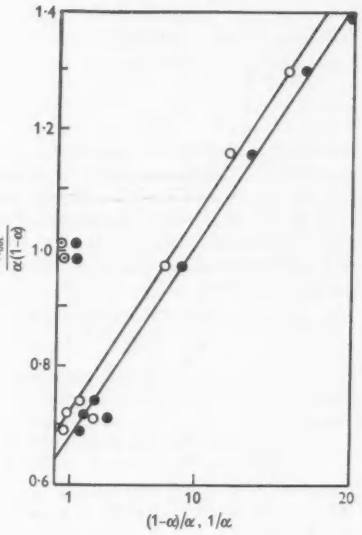

Fig. 8. Plot of $\frac{k_{obs.}}{\alpha(1-\alpha)}$ against $(1-\alpha)/\alpha$ (⊙) and $1/\alpha$ (●) in the case of haemoglobin. (From Brooks's (1935) data.)

discrepancies in the functions plotted. It again appears that the experimental data as a whole do not permit a choice between the two possible equations for $k_{obs.}$.

The empirical equation suggested by Brooks (1931)

$$k_{obs.} = k'\alpha\frac{bp}{1+bp}$$

proved very successful in accounting for the haemoglobin data, but it does not fit the myoglobin data so well as eqns. 4*a* and 4*b* at high oxygen pressures, for Brooks's equation like the single term $k_1\alpha(1-\alpha)$ in eqn. 1 diminishes continuously as p_{O_2} increases. The chief distinction between the haemoglobin and myoglobin systems is that the same range of oxygen pressures, 0·3–760 mm. covers a far wider range in the fraction of unoxygenated haemoprotein in the case of myoglobin than in the case of haemoglobin. This is well brought out by comparison of Tables 2 and 3.

It is probable that other oxygen-pressure functions could be found to account equally well for $k_{obs.}$, particularly if more than one single function is used, as in eqn. 3 above. The merit of the present analysis is that it gives a close fit with the data using only oxygen-pressure functions directly connected with known possible reactants, i.e. α and $(1-\alpha)$ represent the fractions of unoxidized haemoprotein present in the reduced state and in combination with oxygen respectively.

DISCUSSION

The kinetic data on myoglobin and haemoglobin autoxidation reveal the same anomaly at low oxygen pressures. Eqns. 4*a* and 4*b*, 5*a* and 5*b* show that the observed rate constant shows a complex second order variation with oxygen pressure according to the product of the concentrations of Mb and MbO_2, Hb and HbO_2, whilst it is obtained from a first order reaction; for if Fe_p^{2+} represents the total ferrous protoporphyrin concentration and α and $(1-\alpha)$ the fractions of it free and combined with oxygen respectively, then the observed oxygen pressure relationship would be expected to derive from the kinetic equation

$$-\frac{dFe_p^{2+}}{dt} = k(1-\alpha)\ Fe_p^{2+}\ \alpha Fe_p^{2+},$$

i.e. second order in Fe_p^{2+}.

A possible explanation for this anomaly is that the autoxidation is a complicated sequence of reactions, such that the total concentration of unoxidized haemoprotein appears in the denominator of a rate equation of the form

$$-\frac{dFe_p^{2+}}{dt} = \frac{k_a(1-\alpha)\ Fe_p^{2+}\ k_b\alpha Fe_p^{2+}}{k_c Fe_p^{2+}} = \frac{k_a k_b}{k_c}(1-\alpha)\ (\alpha)\ Fe_p^{2+}, \qquad (6)$$

where k_a, k_b and k_c are the rate constants for certain steps in the mechanism. The numerator of the equation suggests that in step a MbO_2 or HbO_2 is involved, giving the term $k_a(1-\alpha)\,Fe_p^{2+}$ and in step b the free Mb or Hb, giving the term $k_b\alpha Fe_p^{2+}$.

This gives an indication as to what may be the operative rate equation at high oxygen pressures, whether it corresponds to 4*a* and 5*a* or 4*b* and 5*b*. As the oxygen pressure is increased Mb and Hb will decrease in concentration and MbO_2 and HbO_2 increase. Thus if both the free and oxygenated forms can undergo the same chemical reaction signified in the rate constant k_b, then the high oxygen pressure relationship would be given by

$$-\frac{dFe_p^{2+}}{dt}=\frac{k_a(1-\alpha)\,Fe_p^{2+}\,k_b'(1-\alpha)\,Fe_p^{2+}}{k_cFe_p^{2+}}, \qquad (7)$$

and the ratio of k_b to k_b' would be given by 2·62/0·30 for myoglobin in eqn. 4*a* and by 0·68/0·04 in the case of haemoglobin in eqn. 5*a*. The fact that, with myoglobin, the activation energy at 760 mm. p_{O_2} is greater than that at 4 mm., as shown in Fig. 5, supports this interpretation, for when MbO_2 reacts additional energy required to break the iron-oxygen bond would be expected to appear as an increase in the activation energy. This is one simple way of accounting for the transition between the kinetics at low and high oxygen pressures. Attempts to account for the order of the reaction and the transition of the kinetics in terms of an equation of the form 4*b* and 5*b* have so far failed, and for this reason interpretation of the kinetics in terms of the rate eqns. 6 and 7 can profitably be explored.

Equations of this type are reminiscent of those derived from free radical mechanisms in which two valency states of a metal ion compete for the same free radical. The mechanism suggested by Weiss (1935) for the autoxidation of ferrous ion, in which the radicals O_2^-, $HO_2^{\cdot}$, H_2O_2 and the OH radical are intermediates, is:

$$Fe^{2+}+O_2 \xrightarrow{1} Fe^{3+}+O_2^-$$
$$O_2^-+H^+ \rightleftharpoons HO_2^{\cdot}$$
$$Fe^{2+}+HO_2 \xrightarrow{2} Fe^{3+}+HO_2^-$$
$$Fe^{3+}+O_2^- \xrightarrow{3} Fe^{2+}+O_2$$
$$HO_2^-+H^+ \rightleftharpoons H_2O_2$$
$$2Fe^{2+}+H_2O_2 \longrightarrow 2Fe^{3+}+2OH^-.$$

From the appropriate stationary state equations it can be shown that the oxidation rate should be

$$-\frac{dFe^{2+}}{dt}=4k_1Fe^{2+}\,O_2\,\frac{k_2Fe^{2+}}{k_2Fe^{2+}+k_3Fe^{3+}}.$$

The numerator in this equation is similar to those in eqns. 6 and 7, but the denominator does not correspond, in fact here the predicted rate is not first order with respect to the ferrous ion concentration. However, a free radical mechanism which can account for the myoglobin and haemoglobin kinetics can be derived in the following way.

Barb, Baxendale, George & Hargrave (1949, 1951), in an extensive study of the ionic iron-hydrogen peroxide system, showed that cupric ions are able to catalyse the reaction of Fe^{3+} with O_2^- through the two steps

$$Cu^{2+}+O_2^- \xrightarrow[\text{determining}]{\text{rate-}} Cu^{+}+O_2,$$
$$Cu^{+}+Fe^{3+} \xrightarrow[\text{rapid}]{\text{very}} Cu^{2+}+Fe^{2+}.$$

Now if it is assumed that there is an auxiliary electron-accepting group in the unoxidized forms of myoglobin and haemoglobin that can act as the cupric ion does, this enables the following reaction scheme to be formulated:

$$0\text{-}Fe_p^{2+}\,O_2 \overset{1}{\underset{3}{\rightleftharpoons}} 0\text{-}Fe_p^{3+}+O_2^-$$
$$0\text{-}Fe_p^{2+}+HO_2 \xrightarrow{2} 0\text{-}Fe_p^{3+}+O_2H^-$$
$$\begin{cases}0\text{-}Fe_p^{2+}\\0\text{-}Fe_p^{2+}\,O_2\end{cases}+O_2^- \xrightarrow{4} \begin{cases}ø\text{-}Fe_p^{2+}\\ø\text{-}Fe_p^{2+}\,O_2\end{cases}+O_2$$
$$\begin{cases}ø\text{-}Fe_p^{2+}\\ø\text{-}Fe_p^{2+}\,O_2\end{cases}+0\text{-}Fe_p^{3+} \xrightarrow[\text{rapid}]{\text{very}} \begin{cases}0\text{-}Fe_p^{2+}\\0\text{-}Fe_p^{2+}\,O_2\end{cases}+0\text{-}Fe_p^{2+}$$

followed by the reaction of the H_2O_2 with two more myoglobin or haemoglobin molecules. In this scheme $0\text{-}Fe_p^{2+}$, $0\text{-}Fe_p^{2+}\,O_2$ and $0\text{-}Fe_p^{3+}$ represent, for instance, Mb, MbO_2 and MetMb with the electron-accepting group in its oxidized state, $ø\text{-}Fe_p^{2+}$ and $ø\text{-}Fe_p^{2+}\,O_2$, when the group in Mb and MbO_2 has accepted an electron. The solution of the stationary state equations gives

$$-\frac{dFe^{2+}}{dt}=\frac{4k_1(1-\alpha)\,Fe_p^{2+}k_2\alpha Fe_p^{2+}}{k_4Fe_p^{2+}+k_2\alpha Fe_p^{2+}+k_3Fe_p^{3+}}.$$

If step 4, which is the back reaction regenerating Mb, predominates, then this equation reduces to

$$-\frac{dFe_p^{2+}}{dt}=\frac{4k_1k_2}{k_4}(1-\alpha)\,(\alpha)\,Fe_p^{2+} \qquad (8)$$

which is identical in form with eqn. 6.

If MbO_2 can also react like Mb in reaction 2 but less rapidly, i.e.

$$0\text{-}Fe_p^{2+}\,O_2+HO_2^{\cdot} \xrightarrow{2'} 0\text{-}Fe_p^{2+}+O_2+O_2H^-,$$

and this reaction replaces reaction 2 at high oxygen pressures, then eqn. 8 becomes

$$-\frac{dFe_p^{2+}}{dt}=\frac{4k_1k_2'}{k_4}(1-\alpha)\,(1-\alpha)\,Fe_p^{2+}, \qquad (9)$$

which is identical with Eqn. 7.

Although this mechanism can account entirely for the kinetic behaviour obtained from spectrophotometric measurements of the metmyoglobin formation, it leaves unexplained the abnormal consumption of oxygen during the reaction. In a previous paper (George & Stratmann, 1952) it was shown that $2{\cdot}5 \pm 0{\cdot}3$ moles of oxygen are used for each mole of metmyoglobin formed. The stoicheiometry in the above mechanism requires the ratio to be 0·25 or 0·5 if the peroxide is utilized in a secondary oxidation. Without detailed knowledge of which groups on the protein molecule are involved no further progress can be made in establishing the precise mechanism. Even though the simple reaction scheme leading to eqns. 8 and 9 is thus inadequate in detail there is good reason to believe it to be correct in principle in so far as the kinetics derive from competition reactions, for such reactions account readily for the form of the rate equation.

SUMMARY

1. The oxidation of myoglobin to metmyoglobin by molecular oxygen at 30° in 0·6M-phosphate buffer, pH 5·69, is shown to be first order in unoxidized myoglobin over a range of oxygen pressures from 0·3 to 760 mm.

2. The observed first order rate constant at first increases with increasing oxygen pressures, shows a well defined maximum value at 1–1·4 mm. partial pressure of oxygen and then decreases to a constant value above 30 mm.

3. The determination of the equilibrium constant for the myoglobin-oxygen reaction under the conditions of the oxidation experiments at 30° gave $K_e = 0{\cdot}88 \pm 0{\cdot}12$ mm.$^{-1}$. Hence the partial pressure for half saturation is 1–1·31 mm. and thus $k_{obs.}$ has its maximum value at half saturation.

4. Using this value of K_e the variation of $k_{obs.}$ with oxygen pressure is shown to be of the form

$$k_{obs.} = 2{\cdot}62\alpha(1-\alpha) + 0{\cdot}30(1-\alpha)^2, \quad (a)$$

or

$$k_{obs.} = 2{\cdot}30\alpha(1-\alpha) + 0{\cdot}30(1-\alpha), \quad (b)$$

where α and $(1-\alpha)$ are the fractions of Mb and MbO_2 respectively.

5. The autoxidation of haemoglobin shows the same kinetic characteristics and Brooks's (1935) data can be represented by the equations:

$$k_{obs.} = 0{\cdot}68\alpha(1-\alpha) + 0{\cdot}04(1-\alpha)^2 \quad (a)$$

or

$$k_{obs.} = 0{\cdot}64\alpha(1-\alpha) + 0{\cdot}04(1-\alpha). \quad (b)$$

6. The activation energy at low oxygen pressures (4 mm.) is 19 ± 1 kg.cal. and at high oxygen pressures (760 mm.) is 25 ± 1 kg.cal. At these extremes the operative form of the equation for $k_{obs.}$ involves only the first and only the last term respectively.

7. Neither the present data for myoglobin nor Brooks's data for haemoglobin permit a choice between equations *a* and *b* but a consideration of the chemical mechanism including the different activation energies at the extremes of oxygen pressure favour equation *a*.

8. A free radical mechanism for the autoxidation is discussed which involves the participation of an auxiliary electron-accepting group on the protein molecule acting as a catalyst in a reaction regenerating the unoxidized haemoprotein and thus serving to 'protect' the haem from oxidation. However, this mechanism is not complete for it does not account for the additional consumption of oxygen above that required for oxidizing the haem group (George & Stratmann, 1952).

The work described in this paper was carried out during the tenure by C.J.S. of an Australian National University Scholarship. We wish to thank Mr T. Greenwood for technical assistance throughout the course of this work.

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Studies on Uridine-Diphosphate-Glucose

By A. C. PALADINI and L. F. LELOIR
Instituto de Investigaciones Bioquímicas, Fundación Campomar, J. Alvarez 1719, *Buenos Aires, Argentina*

(*Received* 18 *September* 1951)

A previous paper (Caputto, Leloir, Cardini & Paladini, 1950) reported the isolation of the coenzyme of the galactose-1-phosphate→glucose-1-phosphate transformation, and presented a tentative structure for the substance. This paper deals with: (*a*) studies by paper chromatography of purified preparations of uridine-diphosphate-glucose (UDPG); (*b*) the identification of uridine-5′-phosphate as a product of hydrolysis; (*c*) studies on the alkaline degradation of UDPG, and (*d*) a substance similar to UDPG which will be referred to as UDPX.

UDPG preparations studied by chromatography. Paper chromatography with appropriate solvents has shown that some of the purest preparations of UDPG which had been obtained previously contain two other compounds, uridinemonophosphate (UMP) and a substance which appears to have the same constitution as UDPG except that it contains an unidentified component instead of glucose. This substance will be provisionally referred to as UDPX (Fig. 1*a*).

The three components have been tested for coenzymic activity in the galactose-1-phosphate→glucose-1-phosphate transformation, and it has been confirmed that UDPG is the active substance. For each mole of uridine of UDPG in a sample extracted from the paper the total phosphate was 2·04, the labile phosphate (15 min. in N-acid at 100°) 1·04, and the reducing power (calc. as glucose) after hydrolysis (10 min. in 0·01 N-acid at 100°) 1·03 moles.

When UDPG is hydrolysed at pH 2 during 10 min. at 100° glucose is liberated and, as shown in Fig. 1*b*, the UDPG and UDPX peaks are replaced by a slow-moving component which is uridinediphosphate.

Fig. 1*c* shows the results obtained after inactivating UDPG with alkali. Besides uridine phosphate a fast- and/or a slow-moving sugar ester are formed.

Identification of uridine-5′-phosphate. The product obtained by hydrolysing off with acid the glucose and one phosphate group from UDPG was previously (Caputto *et al.* 1950) postulated to be uridine-5′-monophosphate. However, the hydrolysis curves of this compound resembled more those given by Gulland & Smith (1947) for uridine-2′-phosphate. Since then Brown, Haynes & Todd (1950) have found that the substance supposed to be uridine-2′-phosphate was uridine-5′-phosphate. The hydrolysis product of UDPG has now been compared with a synthetic specimen of uridine-5′-phosphate. Both substances were found to be identical as judged by chromatographic behaviour (Fig. 1) and by the rate

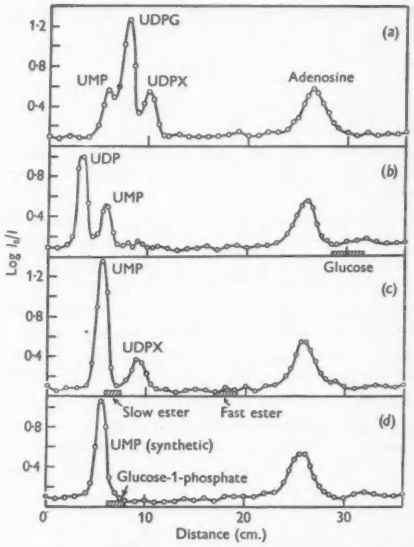

Fig. 1. Chromatograms of UDPG preparations. Samples run simultaneously at 30°. Solvent: ethanol-M-ammonium acetate, pH 7·5. Adenosine was added as reference substance. The log I_0/I values were measured at 260 mμ. *a*, partially purified UDPG; *b*, same after heating 15 min. at 100° at pH 2; *c*, heated 5 min. at 100° with excess NH_4OH; *d*, synthetic uridine-5′-phosphate plus glucose-1-phosphate. Glucose and its esters were located after removing the paraffin by ether extraction followed by spraying with aniline phthalate.

of acid hydrolysis (Table 1). The crystalline barium salts of the two substances were prepared, and after recrystallization from water it was found that the microscopic aspect of both samples was the same. The X-ray diffraction patterns obtained by Prof. Galloni were identical for both samples.

Table 1. *Acid hydrolysis of uridine phosphates*

(Samples heated at 100° in 0·1 N-H_2SO_4.)

Time (hr.)	P hydrolysed (%) Synthetic uridine-5′-phosphate	UMP from UDPG by acid hydrolysis	UMP from UDPG by alkaline hydrolysis
8·2	12·5	13·7	13·7
20·4	26·5	28·2	29·2
36·5	44·4	46·0	43·9
59·5	57·0	59·7	58·7

The alkaline degradation of uridine-diphosphate-glucose. It has been reported previously (Caputto *et al.* 1950) that UDPG loses its catalytic activity after a mild treatment with alkali. It was found that this inactivation was accompanied by a stabilization of the glucose residue and by the liberation of a secondary acid group of phosphoric acid. Further work on this point has shown that mild alkaline treatment of UDPG leads to the formation of UMP and a glucose ester in which the phosphate is doubly esterified. This substance ('Fast Ester') moves faster than any of the known glucose esters during paper chromatography. With a more drastic alkaline treatment or with acid the 'Fast Ester' is transformed into another substance or substances which move more slowly. These are referred to as 'Slow Ester(s)'.

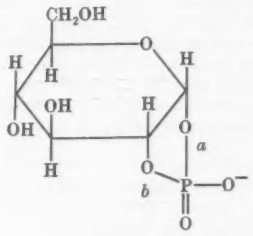

Fig. 2.

The experiments which will be described can be interpreted by assigning to the 'Fast Ester' the structure of a 1:2-monophosphoric ester of glucose (Fig. 2). Further treatment with alkali would yield a mixture of glucose-2- and glucose-1-phosphate by hydrolysis of the links marked *a* and *b* respectively. Treatment with acid would yield the same products, but since glucose-1-phosphate is hydrolysed immediately only glucose-2-phosphate would remain. Thus the 'Slow Ester' prepared with alkali should be a mixture of glucose-1- and glucose-2-phosphates, while that prepared with acid should be glucose-2-phosphate.

The exact conditions under which UDPG is degraded with alkali have not been determined. It is decomposed rapidly during chromatography with the ethanol-ammonia solvent. Under these conditions the formation of the 'Fast Ester' apparently occurs in less than 20 min., since the latter appears as a well defined spot with practically no tailing. At pH 8 at 18° UDPG remained unchanged during 18 hr. At pH 8·5 in 2 min. at 100° a mixture of UMP, and 'Fast' and 'Slow' ester was formed. In concentrated ammonia at 0° during 30 min. UMP and 'Fast Ester' were formed.

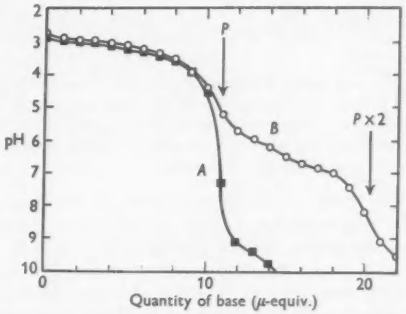

Fig. 3. Titration curve of the 'Fast Ester'. The substance was passed through a column of cation exchange resin in the hydrogen form. One sample was titrated directly (curve *A*) and another sample (curve *B*) was heated 15 min. to 100° before titration. After this treatment 10% of inorganic phosphate and 10% of the glucose were liberated. The arrow marked *P* shows the μmoles of phosphate in the sample.

Properties of the 'Fast Ester'. Table 2 shows the R_F values of the 'Fast Ester' compared with glucose and glucose-1-phosphate. With the solvents which were used the R_F values are grossly inversely proportional to the number of acid groups in the molecule: thus hexosediphosphates move slower than the monophosphates. For the 'Fast Ester' the values

Table 2. *Paper chromatography of the 'Fast' and 'Slow' esters*

(Whatman no. 1 paper.)

Solvent	R_F values 'Fast Ester'	'Slow Ester'	Glucose-1-phosphate	Glucose
Ethanol (77% v/v)	0·29	—	0·10	0·42
Ethanol ammonia	0·53	0·17	0·14	0·58
Ethanol ammonium acetate, pH 7·5	0·55	0·22	0·20	0·71

are nearly as high as those of free glucose. This fact was the first indication that the substance contains fewer acid groups than any of the known hexose-monophosphates. This was confirmed by electrometric titration (Fig. 3) which shows the presence of a primary but no secondary acid group. Acid hydrolysis of the 'Fast Ester' yielded a sugar which was identified as glucose by paper chromatography in several solvents. The same result was obtained after hydrolysis with alkaline phosphatase.

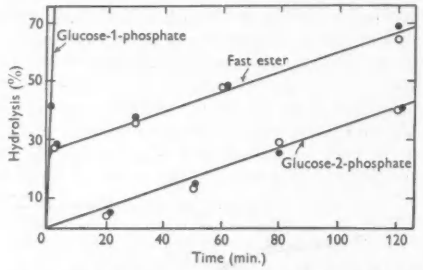

Fig. 4. Acid hydrolysis of the 'Fast Ester'. Samples heated at 100° in 0·1 N-H_2SO_4. Reducing power measured with Somogyi's (Somogyi, 1945) copper reagent followed by arsenomolybdic acid (Nelson, 1944). ●—●, phosphate; ○—○, reducing power as glucose.

The curve of hydrolysis of the 'Fast Ester' in 0·1 N-acid is shown in Fig. 4. The curve shows a break at about 26 % hydrolysis as if it were the curve of a mixture of 26 % glucose-1-phosphate and 74 % glucose-2-phosphate. The curve of liberation of reducing power is parallel to that of phosphate liberation. With the reagent used (Somogyi, 1945) glucose-2-phosphate does not give a detectable reduction. On heating the 'Fast Ester' in 0·1 N-alkali (Fig. 5) 26 % glucose-1-phosphate is formed in less than 5 min. and may be detected with the specific phosphoglucomutase test. The hydrolysis curve of the remaining 74 % of organic phosphate is similar to that of glucose-2-phosphate.

Heating the 'Fast Ester' in dilute acid for a few minutes leads to the liberation of a secondary acid group of phosphoric acid (Fig. 3). The change can also be detected by paper chromatography, since the 'Fast Ester' is transformed into esters having about the same R_F as the normal hexosephosphates (Table 2).

Osazone formation from 'Slow Ester'. The phosphate liberated during osazone formation was estimated on a sample of 'Slow Ester' obtained from the 'Fast Ester' by heating at 100° for 5 min. in 0·1 N-acid. The inorganic phosphate formed by the acid treatment was measured and subtracted from the value obtained after phenylhydrazine treatment. For comparison glucose-1-phosphate and glucose-2-phosphate were also tested. The results were as follows (% liberation of P): glucose-1-phosphate, 0; glucose-2-phosphate, 94 %; 'Slow Ester', 100 %.

Liberation of phosphate during osazone formation would appear in theory to be specific for sugars with a free carbonyl containing phosphate in the 1 or 2 positions. However, it has been observed that glucose-3-phosphate (Raymond & Levene, 1929) and fructose-3-phosphate (Levene, Raymond & Walti, 1929) also lose phosphate during osazone formation.

The structure proposed in Fig. 2 for the 'Fast Ester' is consistent with experiments described in a previous paper (Leloir, 1951), in which a 'Fast

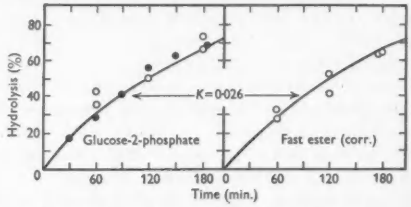

Fig. 5. Alkaline hydrolysis of the 'Fast Ester'. Inorganic phosphate was estimated after the samples (1·1 μM) were heated in 2 ml. of 0·1 N-NaOH to 100° in stoppered bronze tubes. In a parallel experiment glucose-1-phosphate was estimated with yeast phosphoglucomutase (Cardini, Paladini, Caputto, Leloir & Trucco, 1949) activated with glucose diphosphate. Glucose-1-phosphate standards were run at the same time. The samples of the 'Fast Ester' gave 26 % of glucose-1-phosphate after heating 5 min. in 0·1 N-NaOH, and the values remained constant after heating 10 or 20 min. Glucose-1-phosphate was not affected by phosphoglucomutase. The corrected values for the percentage hydrolysis of the 'Fast Ester' were calculated by considering the total phosphate minus the glucose-1-phosphate as equal to 100. ●, Farrar's data; ○, this paper.

Ester' containing galactose was detected besides that containing glucose. Evidence for the α structure of glucose in UDPG has been obtained from preliminary polarimetric observations. It was found that an acid treatment which hydrolysed off the glucose produced a decrease in dextrorotation: $\Delta[M] = 183°$. This value is similar to that for the conversion of α-glucose-1-phosphate to αβ-glucose ($\Delta[M] = 218°$). For an α-glucose ester the likely positions for the formation of a cyclic phosphate would be 1:2 or 1:4. But since 1:4 would be very unlikely for an α-galactose ester it was concluded that both the glucose and the galactose esters were probably esterified at the 1 and 2 positions.

It may be mentioned that Forrest & Todd (1950) have described the formation of a cyclic phosphate of riboflavin by alkaline treatment of flavin-adenine-dinucleotide. Periodate oxidation used 1 mole of oxidant and gave no formic acid so that it was concluded that the phosphate was esterified at positions 4 and 5 of the ribityl residue.

UDPX. The substance giving the small peak which runs faster than UDPG (Fig. 1*a*) has been isolated in small amounts by paper chromatography. Analysis showed that the ultraviolet spectrum at different pH values and after bromine treatment was that of uridine, and that for each mole of uridine in UDPX the total phosphate was 2·04, labile phosphate (15 min. in N-acid at 100°) 1·0, and reducing power after hydrolysis (10 min. in 0·01 N-acid at 100°, calc. as glucose) 0·5 mole.

UDPX was found to remain unaffected by a treatment with alkali sufficient to decompose UDPG (Fig. 1*c*).

The unknown component of UDPX has been studied by chromatography in various solvents, and it has been found to be different from the following substances: aldohexoses, pentoses, fructose, tagatose, sorbose, glucosamine, uronic acids, fucose, rhamnose, xylulose, ribulose, deoxyribose, adonose, erythrulose, 1- and 3-methyl fructose, 2- and 3-methyl glucose, glyceraldehyde and dihydroxyacetone.

UDPX was found to be clearly different from UDP galactose (Leloir, 1951) and from the compound found by Park & Johnson (1949) and Park (1950) in *Staphylococcus aureus*. The R_F values, both of the intact substances and of the sugars obtained by hydrolysis, were different.

The substance X was found to be unfermentable by baker's yeast and to give negative results in the following tests: resorcinol for ketoses (Roe, 1934); Elson & Morgan (1933) for amino sugars, orcinol for pentoses (Mejbaum, 1939), and the test for methyl pentoses (Nicolet & Shinn, 1941). With the aniline-phthalate reagent it gave a brownish-yellow colour which only appeared after prolonged heating.

EXPERIMENTAL

Methods. Analytical methods and preparations were as described in previous papers (Caputto *et al.* 1950). Glucose-2-phosphate was prepared from diphenyl 1:3:4:6-tetra-acetyl-β-D-glucose-2-phosphate (Farrar, 1949) kindly supplied by Mrs K. R. Farrar. A sample of synthetic uridine-5′-phosphate was obtained from Prof. A. R. Todd.

The 'Slow Ester' was usually prepared by paper chromatography of UDPG with ethanol-ammonia as solvent. The position of the substance was revealed in a small part of the paper with aniline phthalate and the ester was subsequently extracted with water.

Paper chromatography. Descending chromatography was usually employed, except when the experiments were carried out in a thermostat. In these cases a more compact chamber similar to that described by Block (1950) was used in which the solvent travels first upwards and then downwards.

Whatman paper no. 1 was employed throughout. For nucleotides and phosphoric esters it was found convenient to wash the paper with 2 N-acetic acid (Hanes & Isherwood, 1949). Usually a pad of blotting paper was stapled at the end of the strips, and the position of the substances was referred to appropriate substances such as glucose for sugars and adenosine for nucleotides. The solvents used were: (*a*) 7·5 vol. of 95% ethanol plus 3 vol. of M-ammonium acetate (pH ~ 7·5); (*b*) same as (*a*) but with M-ammonium acetate buffer of pH 3·8; and (*c*) 7·5 vol. of 95% ethanol plus 3 vol. of concentrated ammonia. With solvent (*a*) the nucleotides give values of $R_{adenosine}$ below 0·7, while the nucleosides give higher values (Table 3). The R_F values of nucleotides vary with the pH of the solvent (Magasanik, Vischer, Doniger, Elson & Chargaff, 1950) and with temperature. As shown in Fig. 6, the changes with temperature are not parallel for all the substances.

Table 3. $R_{adenosine}$ *values, at* $20 \pm 1·5°$ *of some purines, pyrimidines and derivatives*

Substance	Solvent: Ethanol-ammonium acetate, pH 7·5	Solvent: Ethanol-ammonium acetate, pH 3·8
Thymine	—	1·25
Uracil	1·13	1·16
Uridine	1·13	1·16
Cytidine	—	1·10
Adenine	1·06	1·00
Hypoxanthine	—	1·00
Adenosine	1·00	1·00
Uridine-3′-phosphate	0·46	0·94
Guanosine	—	0·88
Cytidylic acid	0·34	0·85
Uridine-5′-phosphate	0·35	0·84
Adenosine-3′-phosphate	0·29	0·76
Adenosine-5′-phosphate	—	0·73
UDPX	0·55	0·73
UDPG	0·43	0·65
Guanylic acid	0·22	0·63
Uridine diphosphate	0·14	0·59
Diphosphopyridinenucleotide	0·21	0·32
Adenosinetriphosphate	0·07	—
Xanthine	0·0	0·0
Guanine	0·0	0·0

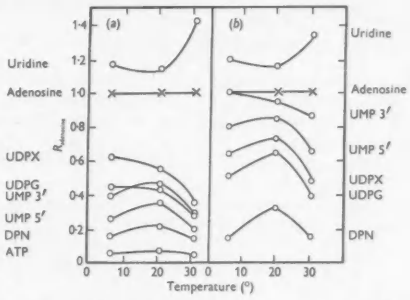

Fig. 6. Paper chromatography of some purine and pyrimidine derivatives. Ethanol-ammonium acetate solvents. *a*, of pH 7·5, and *b*, of pH 3·8, as described in text.

The position of ultraviolet-absorbing substances was ascertained by measuring the extinction at 260 mμ. after impregnation of the paper with liquid paraffin. A standard Beckmann spectrophotometer was used with an accessory

which allowed strips of paper to be run along the photocell entrance (Leloir & Paladini, 1951).

Sugars and their esters were revealed with aniline phthalate reagent (Partridge, 1949).

Liberation of phosphate with phenylhydrazine. This procedure was described by Deuticke & Hollmann (1939) for the estimation of fructosediphosphate. The analytical procedure has been modified by Dr Cardini as follows:

Reagents: (*a*) 6% (w/v) phenylhydrazine hydrochloride in water (decolorized with charcoal if necessary); (*b*) saturated solution of sodium acetate; (*c*) saturated Na_2SO_3.

The samples and phosphate standards in 0·5 ml. of water plus 0·1 ml. of (*a*), 0·05 ml. of (*b*) and 0·1 ml. of (*c*) were heated 30 min. in a boiling-water bath. After cooling 0·75 ml. of 5N-H_2SO_4, 0·75 ml. of 2·5% ammonium molybdate and water to a total vol. 7·5 ml. were added. After 10 min. the optical density was measured at 660 mμ. Controls heated without phenylhydrazine were run at the same time.

SUMMARY

1. Purified preparations of uridine-diphosphate-glucose (UDPG) were studied by paper chromatography and found to be contaminated with uridylic acid and a substance UDPX.

2. The uridylic acid obtained by degradation of UDPG has been identified as uridine-5′-phosphate.

3. The alkaline degradation products of UDPG are uridine-5′-phosphate and a cyclic phosphate ester of glucose, probably esterified at positions 1 and 2 of the glucose. This ester decomposes with acid or alkali giving glucose-1-phosphate (25%) and glucose-2-phosphate (75%).

4. The contaminating substance UDPX appears to have the same structure as UDPG except that it contains an unidentified component instead of glucose.

The studies with synthetic uridine-5′-phosphate and with many samples of rare sugars were possible owing to the kindness of Prof. A. R. Todd, F.R.S., and the identification of glucose-2-phosphate by the generosity of Mrs K. R. Farrar. We wish to express our thanks to them as well as to Prof. E. E. Galloni for the X-ray diffraction studies, to Dr C. E. Cardini for his co-operation with the phenylhydrazine method and to Dr J. T. Park for a sample of the *Staphylococcus aureus* compound.

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The Structure of Urorosein

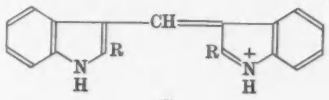
By J. HARLEY-MASON and J. D. BU'LOCK
Chemical Laboratory, University of Cambridge

(*Received* 19 *November* 1951)

Urorosein is a red pigment obtained by the action of mineral acids on the tryptophan oxidation product indole-3-aldehyde (Ellinger & Flamand, 1909). Fearon & Boggust (1950) have reviewed earlier work on the pigment and assigned to it the structure dehydro-indolo-3′:2′:2:3-carbazole. This is the correct systematic name for the structure as shown by Fearon & Boggust and described by them as indolo-3′:2′:2:3-carbazole. It is, however, difficult to see how such a structure containing only a *p*-quinone-diimine chromophore could be so intensely coloured and, moreover, the absorption spectrum as quoted shows a close correspondence to those of the di-3-indolylmethenes described by König (1925). It

seemed desirable, therefore, to re-investigate the structure of this pigment. Fearon & Boggust prepared urorosein sulphate to which they assigned the formula $(C_{18}H_{10}N_2SO_4)_3H_2SO_4$ and further stated that no evidence could be obtained of the splitting off of an aldehyde side chain in the course of formation of the pigment from two molecules of indole-3-aldehyde.

We have repeated the preparation of the sulphate and have made, in addition, the chloride, bromide and perchlorate. Analyses of these salts showed that they are in fact derived from a base of formula $C_{17}H_{12}N$, and it is thus clear that one carbon atom is



(I)

lost in the course of the condensation. We have further shown that this carbon atom is in fact lost as formic acid, which was identified in a distillate of the mother liquors from the preparation of the pigment. Ellinger & Flamand (1909) had earlier shown that 2-methylindole-3-aldehyde was converted by acids into 2:2′-dimethyl-di-3-indolylmethene salts (I; R = Me) with the elimination of formic acid, so that it appeared from our results that indole-3-aldehyde reacted in an exactly similar manner to give di-3-indolylmethene salts (I; R = H). A synthesis of such salts by the reaction of indole with ethyl orthoformate followed by treatment with acids was described by König (1911). We repeated this work and prepared di-3-indolylmethene chloride, bromide, sulphate and perchlorate. These salts were identical in every way with the urorosein salts obtained from indole-3-aldehyde. Melting points were found to be unsatisfactory criteria of identity, since salts all decompose rather indefinitely at temperatures varying with the rate of heating. Identity was therefore established by X-ray powder photographs and absorption spectra. The statement of König (1911), that the sulphate obtained by the orthoformate method and Ellinger & Flamand's urorosein sulphate are spectroscopically identical, is thus confirmed. As Fearon & Boggust observe, the salts tend to dissociate in dilute solution and the absorption spectra were therefore determined in the presence of free acid. The intense colour of the salt is of course due to resonance, the positive charge being shared between the two nitrogen atoms in two equivalent mesomeric structures.

By reaction of ethyl orthoformate for a longer period with excess of indole, a colourless product was obtained; this was tri-3-indolylmethane, which on heating with acids was converted into di-3-indolylmethene salts with elimination of a molecule of indole. Tri-3-(2-methylindolyl)methane has been shown to behave similarly.

In agreement with Fearon & Boggust, we have found that indole-3-acetic acid yields a red pigment, apparently urorosein, on warming with acids in the presence of oxidizing agents, but we have been unable to confirm their statement that a similar pigment is obtained from 2-methylindole.

EXPERIMENTAL

Preparation of urorosein salts

Preparation from indole-3-aldehyde. Finely powdered indole-3-aldehyde (0·5 g.) was heated at 70° for 1 hr. with 35% (w/v) $HClO_4$ (400 ml.) with stirring. After standing overnight the product, which had crystallized partially during the heating, was collected and recrystallized from acetic acid. *Urorosein perchlorate* formed deep-red needles with an intense green reflex and containing a molecule of acetic acid of crystallization. (Found: C, 56·3; H, 4·0; N, 7·0. $C_{17}H_{13}N_2ClO_4.C_2H_4O_2$ requires C, 56·8; H, 4·2; N, 6·95%).

Other salts. Urorosein chloride. This was obtained in the form of deep-red needles from ethanol-acetone. (Found: C, 72·3; H, 4·9; Cl, 12·4. $C_{17}H_{13}N_2Cl$ requires C, 72·7; H, 4·6; Cl, 12·6%.)

The *bromide* crystallized in the form of deep-red prisms from ethanol. (Found: C, 62·4; H, 4·3; Br, 24·6. $C_{17}H_{13}N_2Br$ requires C, 62·8; H, 4·0; Br, 24·5%.) *Urorosein sulphate* formed deep-red plates from acetic acid. (Found: C, 60·1; H, 4·0; N, 8·0. $C_{17}H_{13}N_2.HSO_4$ requires C, 59·8; H, 4·1; N, 8·2%.) These salts were prepared exactly similarly to the perchlorate using 20% (w/v) HCl, 20% (w/v) HBr and 30% (w/v) H_2SO_4.

Preparation from indole and ethyl orthoformate. Indole (1·2 g.) and ethyl orthoformate (0·8 g.) were dissolved in ethanol (5 ml.), 1 drop of dilute ethanolic HCl added as catalyst and the mixture refluxed for 1 hr. After cooling, the deep-yellow solution was diluted with ethanol (50 ml.) and then 20% (w/v) $HClO_4$ (50 ml.) added with stirring. Urorosein perchlorate crystallized out and was collected and recrystallized as above. The sulphate, chloride and bromide were prepared similarly using the appropriate acids in place of $HClO_4$.

The ultraviolet absorption spectra, determined with a Beckmann spectrophotometer, of the bromides and perchlorates prepared by the two methods above in 95% ethanol containing 5% HBr and $HClO_4$ respectively were compared and found to be identical, having maxima at 258, 286, 487 and 530 mμ. X-ray powder photographs of the bromides prepared by the two methods were identical.

The action of NH_3 on urorosein salts gave the yellow free base in crystalline form: the material was, however, unstable and could not be obtained analytically pure.

Tri-3-indolylmethane

Indole (2 g.) and ethyl orthoformate (0·8 g.) were dissolved in ethanol (7 ml.) one drop of ethanolic HCl added and the mixture refluxed for 6 hr. Towards the end of the heating some pale-yellow crystalline material began to separate. After cooling the solid was filtered off and recrystallized from ethanol. *Tri-3-indolylmethane* formed

colourless prisms, m.p. 244–246°. (Found on a sample dried at 100°/10 mm.: C, 83·2; H, 5·0; N, 11·6. $C_{25}H_{19}N_3$ requires C, 83·0; H, 5·25; N, 11·6%.)

Detection of formic acid

Indole-3-aldehyde (0·2 g.) was treated with 10% (w/v) HCl (100 ml.) at 60° for 20 min. and the urorosein chloride filtered off. The mother liquor was distilled at atmospheric pressure and formic acid was detected in the first portion of the distillate by reduction to formaldehyde with Mg and reaction with chromotropic acid (Feigl, 1946).

SUMMARY

Urorosein salts are shown to be derived from di-3-indolylmethene and can be prepared either from indole-3-aldehyde or from indole and ethyl orthoformate.

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